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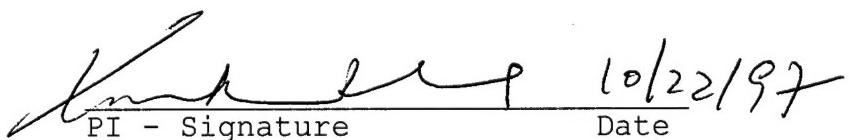
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## **TABLE OF CONTENTS**

	<b>Page</b>
Front Cover.....	1
Report Documentation SF 298.....	2
Foreword.....	3
Table of Contents.....	4
Introduction.....	5
Body of report.....	5-10
Conclusions.....	10
References.....	11-12
Appendix .....	12

## INTRODUCTION

Retinoids, retinoic acid (RA) and its analogs, are promising chemopreventive agents against breast cancer. Their efficacy against mammary carcinogenesis in animal models has been demonstrated by their ability to increase the latency period for tumor appearance and decrease the number of animals with cancer (1). Despite their cancer preventive effects, retinoids tested in clinical trials have not yet caused major clinical responses (2, 3), implying that their anti-cancer effectiveness diminishes in more malignant cells and leads to one of the major drawbacks in retinoid therapy, the retinoid resistance. The ineffectiveness of retinoids in the treatment of patients with advanced breast cancer is consistent with in vitro observations that the anti-cancer effects of retinoic acid (RA) are mainly seen in estrogen-dependent breast cancer cells and that, upon progression of the disease to estrogen independence, breast cancer cells become refractory to RA.

The effects of retinoids are mainly mediated through the three types ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) of the RA receptors (RARs) and retinoid X receptors (RXRs), which function *in vivo* mainly as RXR/RAR heterodimers (4-6). The RXR/RAR heterodimers are activated by RAR ligands. Binding of RXR ligands can induce RXR homodimer formation and function (6) and may be required for activation of certain RXR-containing heterodimers, such as RXR/LXR (7) and RXR/nur77 (8, 9). The dimeric complexes of retinoid receptors function as transcriptional factors that bind to specific sequences on target genes and regulate the transcriptional expression of the genes. One of the most potent target genes identified so far is RAR $\beta$  gene. RAR $\beta$  is activated by RA through a RA response element ( $\beta$ RARE) in its promoter (10). The auto-induction of RAR $\beta$  expression may play a critical role in amplifying retinoid responses.

Regulation of gene expression either positively or negatively by nuclear hormone receptors is modulated by additional cofactors that appear to provide a direct link to the core transcriptional machinery and to modulate chromatin structure (11). Some of these cofactors (coactivators) enhance transactivation by several nuclear receptors in the presence of their cognate ligands, whereas others (corepressors) are necessary for unliganded receptors to silence the activity of target promoters, and are dissociated upon binding of ligand to the receptors. In addition to receptor coactivators and corepressors, a number of other cellular proteins, such as AP-1, have been implicated in the regulation of nuclear hormone receptor activity, probably through their interaction with receptors (12).

Despite intensive research, the molecular mechanisms by which retinoids exert their anti-cancer effects and how their activities are lost in certain breast cancer cells remain unclear. The major goal of the proposed experiments is to understand the molecular mechanism by which retinoids exert their anti-cancer effects in breast cancer cells. The specific aims of this project are: 1) to analyze the anti-estrogen effect of retinoid receptors; 2) to characterize proteins that interact with RXR; 3) to analyze transactivation and anti-AP-1 activities of retinoid receptors; 4) to analyze the mechanism by which retinoid receptor activities are impaired, and 5) to analyze the function of RXR homodimers.

## BODY

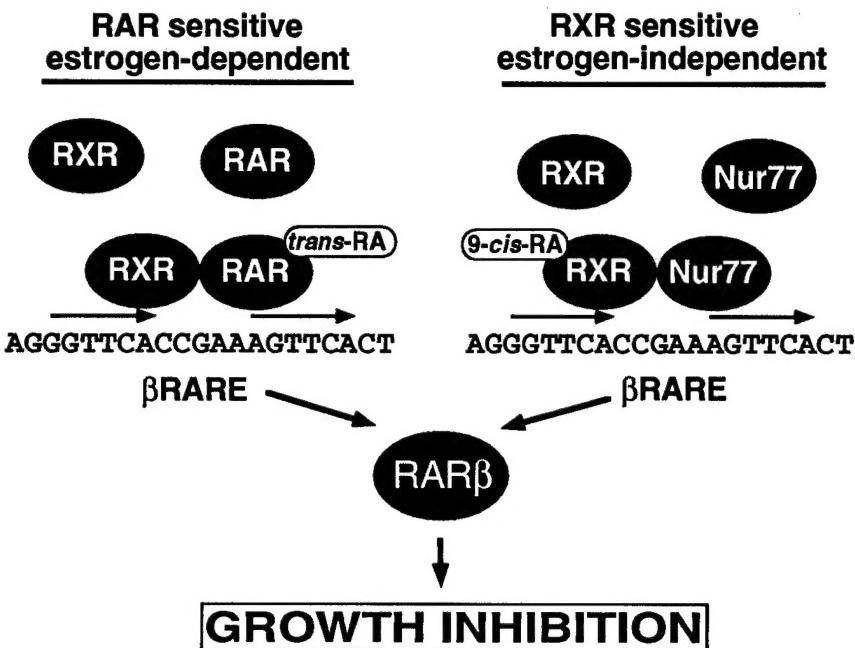
**RXR-selective retinoids inhibits the growth of *trans*-RA-resistant estrogen-independent breast cancer cell lines (Wu et al., Mol. Cell. Biol. in press, 1997).** We showed previously that activation of RARs, but not RXRs is required for growth inhibition in estrogen-dependent ZR-75-1 cells (13). To further investigate the role of RARs and RXRs in breast cancer cells, we used a number of RAR-selective and RXR-selective retinoids to determine their growth inhibitory effects in both *trans*-RA-sensitive, estrogen-dependent and *trans*-RA-resistant, estrogen-independent cells. RAR-selective retinoids strongly inhibited the growth of *trans*-RA-sensitive ZR-75-1 cells, but not *trans*-RA-resistant cells. In contrast, RXR-selective retinoids did not affect *trans*-RA-sensitive cell growth, but significantly inhibited the growth of *trans*-RA resistant MDA-MB-231 cells. RXR-selective retinoid

also induced apoptosis in *trans*-RA-resistant cells but not in *trans*-RA-sensitive cells, whereas RAR-selective but not RXR-selective retinoid induced apoptosis only in ZR-75-1 cells. These data suggest that the RXR pathway is involved in *trans*-RA-resistant breast cancer cell growth inhibition and apoptosis. The combination of either RXR-selective retinoid with the RAR-selective retinoid strongly enhanced the growth inhibitory effect of the RXR-selective retinoids. Thus, different retinoid signaling pathways can mediate retinoid-induced growth inhibition in estrogen-dependent and -independent breast cancer cells.

**Expression of RAR $\beta$  correlates with growth inhibitory effect of RXR-selective retinoids (Wu et al., Mol. Cell. Biol. in press, 1997).** We showed previously that expression of RAR $\beta$  can mediates the growth inhibitory effects of RA (13). To determine whether the growth inhibitory effects of RXR-selective retinoids are also mediated by expression of RAR $\beta$ , we analyzed RAR $\beta$  gene expression in both estrogen-dependent and -independent breast cancer cell lines in response to RAR-selective and RXR-selective retinoids. RAR-selective retinoids strongly induced RAR $\beta$  expression in *trans*-RA-sensitive ZR-75-1 and T-47D cells, while RXR-selective retinoids did not. In contrast, both RAR-selective and RXR-selective retinoids could slightly induce RAR $\beta$  in *trans*-RA-resistant MDA-MB-231 cells. The combination of RAR-selective and RXR-selective retinoids induced RAR $\beta$  to a level comparable to that observed in T-47D cells. Thus, our data demonstrate that RAR $\beta$  induction and activation correlates with growth inhibition and suggest that RAR $\beta$  induction may contribute to growth inhibition by RXR-selective retinoids.

**Induction of RAR $\beta$  by RXR-selective retinoids through RXR/nur77 heterodimers (Wu et al., Mol. Cell. Biol. in press, 1997).** The  $\beta$ RARE in the RAR $\beta$  gene promoter mediates RAR $\beta$  expression (10). The  $\beta$ RARE is activated by the RXR/RAR heterodimer in response to RAR-selective retinoids, but not RXR-selective retinoids (8). Recently, orphan receptor nur77 was shown to bind to DR-5 type RARE as RXR/nur77 heterodimers (8, 9). We investigated the binding of nur77 on the  $\beta$ RARE. Nur77 alone did not show clear binding, but when mixed with RXR $\alpha$ , strong binding to  $\beta$ RARE occurred (14,15). Thus, the  $\beta$ RARE is an unique RARE that allows interaction with both RXR/nur77 and RXR/RAR heterodimers. To determine whether binding of the RXR/nur77 heterodimers to the  $\beta$ RARE could activate the response element in response to RXR-selective retinoids, we performed a transient transfection assay (15). Cotransfection of nur77 and RXR expression vectors significantly enhance the  $\beta$ RARE activity in response to RXR-selective retinoids. This data suggests that induction of RAR $\beta$  by RXR-selective retinoids through RXR/nur77 heterodimers may be the mechanism by which RXR-selective retinoids inhibit the growth of MDA-MB-231 cells.

**Levels of RAR $\alpha$  modulates retinoid signaling switch in breast cancer cells (Wu et al., Mol. Cell. Biol. in press, 1997).** Many estrogen-dependent and -independent breast cancer cell lines have similar expression levels of RAR $\gamma$  and RXR $\alpha$  (13). Nur77 expression levels are similar in ZR-75-1 and MDA-MB-231 cells. However, RAR $\alpha$  is only highly expressed in the estrogen-dependent, *trans*-RA-sensitive breast cancer cell lines (15). This suggests the possibility that expression of RAR $\alpha$  may allow preferential formation of RAR $\alpha$ /RXR heterodimers in estrogen-dependent breast cancer cell lines, that function to mediate the growth inhibitory effect of RAR-selective retinoids but not RXR-selective retinoids. In contrast, low expression level of RAR $\alpha$  in estrogen-independent, *trans*-RA-resistant breast cancer cell lines may permit formation RXR/nur77 heterodimers that can be activated by RXR-selective retinoids to induce RAR $\beta$ . When we stably express RAR $\alpha$  in MDA-MB-231 cells to enhance the level of RAR $\alpha$ , the effects of RAR-selective retinoids on growth inhibition and RAR $\beta$  induction was enhanced while the effect of RXR-selective retinoids was suppressed. The low RAR $\alpha$  level in MDA-MB-231 cells (13) favors nur77/RXR formation that activates the  $\beta$ RARE in response to RXR-selective retinoids. Therefore, depending on the RAR, RXR, and nur77 levels present in the cancer cells, either the *trans*-RA- or 9-*cis* RA-signaling pathway can activate the  $\beta$ RARE (Figure 1). This retinoid signaling switch may play an important role in regulating cell growth in response to different stimuli and suggests that low expression of RAR $\alpha$  may allow 9-*cis*-RA signaling but cause *trans*-RA resistance.



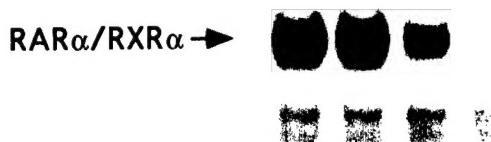
**Figure 1.** Retinoid signaling pathways in breast cancer cells. RAR $\beta$  expression can mediate the growth inhibitory effect of retinoids and is regulated by the βRARE in RAR $\beta$  promoter. βRARE bound to both RXR $\alpha$ /RAR $\alpha$  and RXR $\alpha$ /nur77. Relative cellular levels of RAR, RXR and nur77 may determine whether βRARE binds to RXR $\alpha$ /RAR $\alpha$  or RXR $\alpha$ /nur77. Estrogen-dependent, *trans*-RA-sensitive breast cancer cells expressed higher levels of RAR $\alpha$  to favor binding of RXR $\alpha$ /RAR $\alpha$  that can be activated by RAR-selective ligands to induce RAR $\beta$  expression. In contrast, estrogen-independent, *trans*-RA-resistant breast cancer cells expressed lower levels of RAR $\alpha$  to favor RXR $\alpha$ /nur77 formation, which bound to the βRARE. RXR-selective retinoids activate nur77/RXR to induce RAR $\beta$  expression. Induction of RAR $\beta$  by either RAR- or RXR-selective retinoids could lead to growth inhibition.

**Heterodimerization of orphan receptors COUP-TF and nur77 (Wu et al., EMBO J. 16: 1656-1669, 1997).** In the course to investigate the effect of nur77 in CV-1 cells, we observed that nur77 could inhibit COUP-TF binding on the βRARE. COUP-TF formed a strong complex to the βRARE. However, when nur77 protein was added, the COUP-TF binding complex was inhibited. Conversely, the binding of RXR/nur77 on the βRARE was efficiently inhibited by either COUP-TFI or COUP-TFII. Our immunoprecipitation assay using anti-nur77 antibody and other protein-protein interaction assays demonstrate that nur77 forms a stable complex with COUP-TFI or COUP-TFII in solution. These observations suggest that nur77 not only can mediate the effect of RXR-ligands through its heterodimerization with RXR but also can regulate retinoid sensitivity through its interaction with COUP-TF that binds to the βRARE. However, nur77 is expressed at relative low levels in breast cancer cell lines. It may mainly function as RXR/nur77 heterodimers to mediate the effect of RXR-ligands in estrogen-independent breast cancer cell lines since these cell lines do not express COUP-TF. Nevertheless, the relationship between nur77, COUP-TF and RXR in breast cancer cell lines remains to be determined.

**Down-regulation of AP-1 transcriptional activation in breast cancer cells by retinoids (Agadir et al., Cancer Res. 57: 3444-3450, 1997).** Retinyl methyl ether (RME) is a well known inhibitor of mammary cancer development (16,17). However, the mechanism by which RME exerts its anti-cancer effects is presently unclear. In our previous study (18), we have demonstrated that RME can modulate transactivational activity of retinoid receptors on RAREs. In this study, we investigate whether RME can down-regulate transcriptional activation by the tumor promoter TPA, growth factor

and the nuclear protooncogenes cJun and cFos. Transient transfection assays demonstrate that RME can repress transcriptional induction by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate TPA, EGF, insulin, cJun and cFos of the human collagenase promoter or a heterologous promoter that contains the AP-1 binding site. In HeLa cells, the inhibition was observed when RAR $\alpha$  expression vector was cotransfected, while the endogenous retinoid receptors in breast cancer cells are sufficient to confer the inhibition by RME. In addition, the expression of nur77 induced by TPA and growth factor could be inhibited by RME. These results suggest that inhibition of AP-1 responsive genes may represent an important mechanism by which RME prevents cancer development.

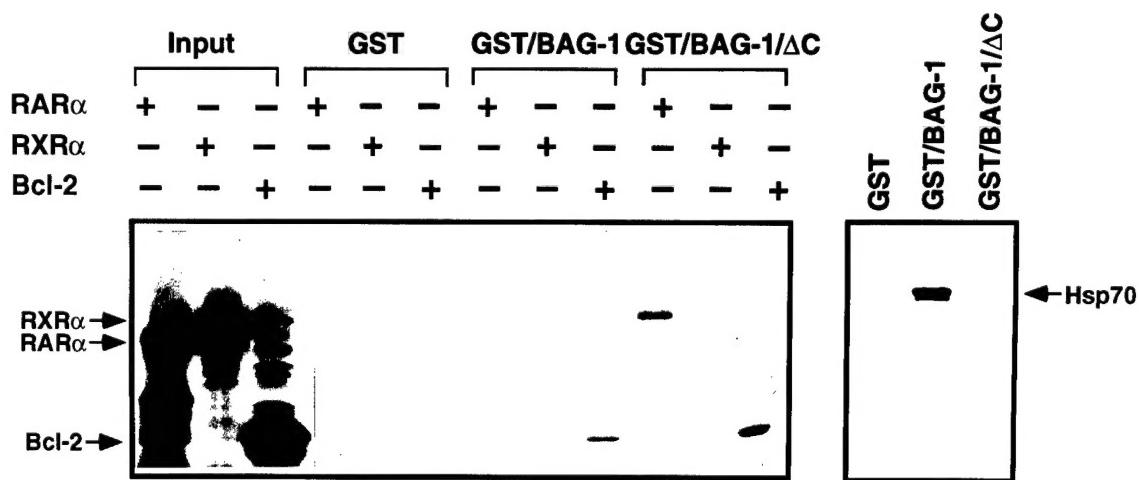
BAG-1:	-	1	2	5
RAR $\alpha$ /RXR $\alpha$ :	+	+	+	+



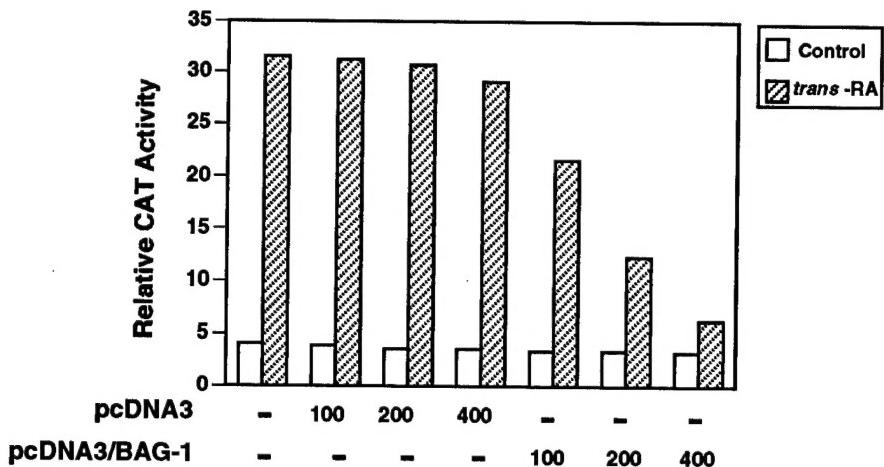
**Figure 2. Inhibition of RAR DNA binding by BAG-1.** In vitro synthesized RAR $\alpha$  and RXR $\alpha$  were preincubated with the indicated molar excess of in vitro synthesized BAG-1. Unprogrammed reticulocyte lysate was used to maintain an equal protein concentration in each reaction. Following this pre-incubation, the reaction mixtures were incubated with  $^{32}$ P-labeled  $\beta$ RARE and analyzed by the gel retardation assay.

**Interaction of BAG-1 with retinoic acid receptor and its inhibition of retinoic acid-induced apoptosis in breast cancer cells (Liu et al., submitted, 1997)** BAG-1 (also known as RAP46) is an anti-apoptotic protein. It was cloned from a murine embryo cDNA library using a protein-protein interaction technique (19). Co-expression of BAG-1 and Bcl-2 in Jurkat lymphoid cells, NIH 3T3 fibroblasts, and melanoma cells promoted the survival of these cells in response to a variety of apoptotic stimuli (19). In addition to Bcl-2, BAG-1 also interacts with Raf-1 (20), and can activate this kinase through a Ras-independent mechanism. Furthermore, BAG-1 can interact with hepatocyte growth factor (HGF) receptor and with platelet-derived growth factor (PDGF) receptor, and enhance the ability of these receptors to transduce signals for cell survival (21). These observations suggest that BAG-1 may function as an adaptor to mediate the interaction between survival factors and apoptotic machinery, and may also play a role in regulating cellular proliferation. Interestingly, the human BAG-1 homolog (also known as RAP46) was cloned from a human liver cDNA library by virtue of its interaction with the

glucocorticoid receptor (22). In vitro, RAP46 interacts with a number of nuclear hormone receptors, including estrogen receptor (ER) and thyroid hormone receptor (TR) (22). Recently, it has been shown that BAG-1 binds tightly to Hsp70/Hsc70-family proteins and modulates their chaperone activity (23). Since molecular chaperones are known to play an important role in controlling the activity of many members of the steroid/thyroid/retinoid receptor family (24), it is possible that BAG-1 could alter the function of these transcriptional regulators. Prior to this report, however, it was unknown whether BAG-1 regulates the activities of the nuclear hormone receptors and whether BAG-1 interacts with retinoid receptors. We studied whether BAG-1 interacts with RAR. Gel retardation assays demonstrated that in vitro translated BAG-1 protein could effectively inhibit the binding of RAR/RXR to the  $\beta$ RARE (Figure 2). A glutathione S-transferase (GST)-BAG-1 fusion protein also specifically bound RAR but not RXR (Figure 3). Interaction of BAG-1 and RAR could also be demonstrated by yeast two-hybrid assays. In transient transfection assays, co-transfection of BAG-1 expression plasmid inhibited the transactivation activity of RAR/RXR heterodimers (Figure 4). When stably expressed in both MCF-7 and ZR-75-1 breast cancer cell lines, BAG-1 suppressed retinoic acid (RA)-induced growth inhibition and apoptosis. In addition, RA-induced suppression of Bcl-2 expression was abrogated by over-expression of BAG-1. Taken together, our results demonstrate that BAG-1 can physically interact with RARs and is an important component of the retinoid response pathway. The findings suggest that this protein-protein interaction may play an important role in the regulation of retinoid-induced growth inhibition and apoptotic processes, potentially contributing to retinoid resistance in cancer.



**Figure 3. Analysis of RAR-BAG-1 interaction by the GST pull-down assay.** BAG-1 or a BAG-1 C-terminal deletion mutant (BAG-1/ $\Delta$ c) was expressed in bacteria using the pGex.4T expression vector. The GST-BAG-1 proteins were immobilized on glutathione-Sepharose beads. As a control, the same amount of GST was also immobilized.  $^{35}$ S-labeled RAR $\alpha$ , RXR $\alpha$  or Bcl-2 was then mixed with the beads. After extensive washing, the bound proteins were analyzed by SDS-PAGE. The input proteins are shown for comparison (left panel). For comparison, binding of GST-BAG-1 and GST-BAG-1/ $\Delta$ c to Hsc70 was shown in right panel.



**Figure 4. Inhibition of trans-activation activities of nuclear receptors by BAG-1.** The TREPAL-tk-CAT reporter plasmid was co-transfected into CV-1 cells with 100 ng RAR $\alpha$  expression vectors together with the indicated amounts of BAG-1 expression vector (pcDNA3/BAG-1) or the empty plasmid (pcDNA3) into CV-1 cells. Transfected cells were treated with or without  $10^{-7}$  M *trans*-RA, and assayed 24 h later for CAT activity.

## CONCLUSION

Research conducted in the past funding year shows several pieces of exciting findings. We have now convincingly demonstrated that activation of RXR by RXR-selective retinoids can induce growth inhibition in estrogen-independent, *trans*-RA-resistant breast cancer cells. In addition, a synergistic growth inhibition effect was observed with combination of certain RAR-selective and RXR-selective retinoids. The effect of RXR-selective retinoids is mediated through RXR/nur77 heterodimers that bind to the  $\beta$ RARE and activate RAR $\beta$  gene expression in response to RXR ligands. This finding is important in that we demonstrate a novel growth inhibition pathway that is mediated by activation of RXR. Importantly, the pathway is functional in RAR $\alpha$ -negative, estrogen-independent and *trans*-RA-resistant breast cancer cells. Thus, different retinoid growth inhibition pathways exist and different classes of retinoids can be used for different types of breast cancer. The combination treatment using a RAR-selective retinoid and a RXR-selective retinoid that synergizes could provide a means of reducing the effective dose and toxicity of retinoids. We also show that inhibition of AP-1 activity may represent another mechanism by which retinoids prevent mammary cancer development. Furthermore, we observed that retinoid receptors could interact with a cell survival gene, BAG-1, suggesting that BAG-1 can regulate retinoid activity through its interaction with RARs and may mediate the interaction between retinoid signaling and growth and apoptosis signalings. As proposed, in the coming year we will further investigate the molecular mechanism by which retinoid receptors function in breast cancer cells. Due to our finding of the involvement of orphan receptors nur77 and COUP-TF in regulating retinoid activity, our research will focus on the mechanism by which RXR/nur77 heterodimers mediate the effect of RXR-selective retinoids and the involvement of COUP-TF in regulating the activity of RXR/RAR and RXR/nur77 heterodimers. In addition, we will further investigate the mechanism by which BAG-1 interacts with RARs and regulates retinoid activities in breast cancer cells, including the involvement of heat shock proteins.

## REFERENCES

1. Moon, R. C., Mehta, R. G., and Rao, K.V.N. Retinoids and cancer in experimental animals. In *The Retinoids: Biology, Chemistry, and Medicine*, 2nd ed. M. B. Sporn, A. B. Roberts, and D. S. Goodman (Eds.), Raven Press, New York, 1994, pp. 573-595.
2. Cassidy, J., Lippman, M., LaCroix, A., et al. Phase II trial of 13-cis-retinoic acid in metastatic breast cancer. *Eur. J. Cancer Clin. Oncol.* **18**: 925-928, 1982.
3. Modiano, M., Dalton, W., Lippman, S., et al. Phase II study of fenretinide (N-[4-hydroxyphenyl]retinamide) in advanced breast cancer and melanoma. *Invest. New Drugs* **8**: 317-319, 1990.
4. Mangelsdorf, D. J., and Evans, R. M. The RXR heterodimers and orphan receptors. *Cell* **83**: 841-850, 1995.
5. Kastner, P., Mark, M., and Chambon, P. Nonsteroid nuclear receptors: What are genetic studies telling us about their role in real life? *Cell* **83**:859-869, 1995.
6. Zhang, X-k., and Pfahl, M. Regulation of retinoid and thyroid hormone action through homodimeric and heterodimeric receptors. *Trends Endocrinol. Metab.* **4**: 156-162, 1993.
7. Willy, P. J., Umesono, K., Ong, E. S., Evans, R. M., Heyman, R. A. and Mangelsdorf, D. J. LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Dev.* **9**:1033-45, 1995.
8. Forman, B. M., Umesono, K., Chen, J., and Evans, R. M. Unique response pathways are established by allosteric interactions among nuclear hormone receptors. *Cell* **81**: 541-550, 1995.
9. Perlmann, T., and Jansson, L. A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and Nurr1. *Genes Dev.* **9**: 769-782, 1995.
10. Hoffmann, B., Lehmann, J. M., Zhang, X-k., Hermann, T., Graupner, G., and Pfahl, M. A retinoic acid receptor-specific element controls the retinoic acid receptor- $\beta$  promoter. *Mol. Endocrinol.* **4**: 734-1743, 1990.
11. Horwitz,K.B., Jackson,T.A., Bain,D.L., Richer,J.K., Takimoto,G.S. and Tung,L. Nuclear receptor coactivators and corepressors. *Mol. Endocrinol.*, **10**, 1167-1177, 1996.
12. Pfahl,M. Nuclear receptor/AP-1 interaction. *Endocr. Rev.*, **14**: 651-658, 1993.
13. Liu, Y., Lee, M.-O., Wang, H.-G., Li, Y., Hashimoto, Y., Klaus, M., Reed, J. C., and Zhang, X.-k. RAR $\beta$  mediates the growth inhibitory effect of retinoic acid by inducing cell apoptosis in human breast cancer cells. *Mol. Cell. Biol.* **16**: 1138-1149, 1996.
14. Wu,Q., Li,Y., Liu,R., Agadir,A., Lee,M.-O., Liu,Y. and Zhang,X.-k. Modulation of retinoic acid sensitivity in lung cancer cells by a dynamic balance of nur77 and COUP-TF orphan receptors and their heterodimerization. *EMBO J.*, **16**: 1656-1669, 1997.
15. Wu,Q., Dawson,M.I., Zheng,Y., Hobbs,P.D., Agadir,A., Jong,L., Li,Y., Liu,R., Lin,B. and Zhang,X.-k. Inhibition of trans-retinoic acid-resistant human breast cancer cell growth by retinoid x receptor-selective retinoids. *Mol. Cell. Biol.* in press, 1997.
16. Grubbs, C.J., Moon, R.C., Sporn, M.B., and Newton, D.L. Inhibition of mammary cancer by retinyl methyl ether. *Cancer Res.* **37**: 599-602, 1977.

17. Mossman, B.T., Craighead, J.E., and MacPherson, B.V. Asbestos-induced epithelial changes in organ cultures of hamster trachea: inhibition by retinyl methyl ether. *Science* **207**: 311-313, 1980.
18. Sani, B.P., Zhang, X.-k., Hill, D.L., and Shealy, Y.F. Retinyl ethers: binding properties, chemopreventive potential, and transcriptional regulation. *Biochem. Biophys. Res. Comm.* **223**: 293-298, 1996.
19. Takayama,S., Sato,T., Krajewski,S., Kochel,K., Irie,S., Millan,J.A. and Reed,J.C. Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. *Cell* **80**: 279-284, 1995.
20. Wang,H.-G., Takayama,S., Rapp,U.R. and Reed,J.C. Bcl-2 interacting protein, BAG-1, binds to and activates the kinase Raf-1. *Proc. Natl. Acad. Sci. USA* **93**: 7063-7068, 1996.
21. Bardelli,A., Longati,P., Albero,D., Goruppi,S., Schneider,C., Ponzetto,C. and Comoglio,P.M. HGF receptor associates with the anti-apoptotic protein BAG-1 and prevents cell death. *EMBO J* **15**: 6205-6212, 1996.
22. Zeiner,M. and Gehring,U. A protein that interacts with members of the nuclear hormone receptor family: identification and cDNA cloning. *Proc. Natl. Acad. Sci. USA* **92**: 11456-11469, 1995.
23. Takayama,S., Bimston,D.N., Matsuzawa,S., Freeman,B.C., Aime-Sempe,C., Xie,Z., Morimoto,R.I. and Reed,J.C. BAG-1 modulates the chaperone activity of Hsp70/Hsc70. *EMBO J.* **16**: 4887-4896, 1997.
24. Pratt,W.B. and Toft,D.O. Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr. Rev.* **18**: 306-360, 1997.

## APPENDIX

1. Wu, Q., Li, Y., Liu, R., Agadir, A., Lee, M.-O., Liu, Y., and Zhang, X.-k. Modulation of retinoic acid sensitivity in lung cancer cells through dynamic balance of orphan receptors nur77 and COUP-TF and their heterodimerization . *EMBO J.* **16**: 1656-1669.
2. Agadir, A., Shealy, Y.F., Hill, D.L., and Zhang, X.-k. Retinyl methyl ether down-regulates activator protein 1 transcriptional activation in breast cancer cell. *Cancer Res.* **57**: 3444-3450, 1997.
3. Wu, Q., Cheng, Y., Dawson, M.I., Hobbs, P.D., Li, Y., and Zhang, X.-k. Inhibition of trans-RA-resistant human breast cancer cell growth by retinoid X-receptor-selective retinoids. *Mol. Cell. Biol.* In Press, 1997
4. Liu,R., Takayama, S., Zheng, Y., Froesch, B., Chen, G-q., Zhang, X., Reed, J.C. and Zhang, X.-k. Interaction of BAG-1 with retinoic acid receptor and its inhibition of retinoic acid-induced apoptosis in breast cancer cells. (Submitted, 1997).

# Modulation of retinoic acid sensitivity in lung cancer cells through dynamic balance of orphan receptors nur77 and COUP-TF and their heterodimerization

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**The diverse function of retinoic acid (RA) is mediated by its nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). However, the RA response is often lost in cancer cells that express the receptors. Previously, it was demonstrated that the RA response is regulated by the COUP-TF orphan receptors. Here, we present evidence that nur77, another orphan receptor whose expression is highly induced by phorbol esters and growth factors, is involved in modulation of the RA response. Expression of nur77 enhances ligand-independent transactivation of RA response elements (RAREs) and desensitizes their RA responsiveness. Conversely, expression of COUP-TF sensitizes RA responsiveness of RAREs by repressing their basal transactivation activity. Unlike the effect of COUP-TFs, the function of nur77 does not require direct binding of nur77 to the RAREs, but is through interaction between nur77 and COUP-TFs. The interaction occurs in solution and results in inhibition of COUP-TF RARE binding and transcriptional activity. Unlike other nuclear receptors, a large portion of the carboxy-terminal end of nur77 is not required for its interaction with COUP-TF. In human lung cancer cell lines, COUP-TF is highly expressed in RA-sensitive cell lines while nur77 expression is associated with RA resistance. Stable expression of COUP-TF in nur77-positive, RA-resistant lung cancer cells enhances the inducibility of RAR $\beta$  gene expression and growth inhibition by RA. These observations demonstrate that a dynamic equilibrium between orphan receptors nur77 and COUP-TF, through their heterodimerization that regulates COUP-TF RARE binding, is critical for RA responsiveness of human lung cancer cells.**

**Keywords:** lung cancer/orphan receptors/receptor dimerization/retinoic acid receptors/retinoic acid sensitivity

## Introduction

Retinoic acid (RA) and its natural and synthetic vitamin A derivatives, retinoids, are known to regulate a broad range of biological processes, and are used currently in the treatment of epithelial cancer and promyelocytic leukemia (Gudas *et al.*, 1994; Hong and Itri, 1994).

However, retinoid resistance associated with many different types of cancer has prevented retinoids from further application (Warrell *et al.*, 1993; Hong and Itri, 1994). The effects of retinoids are mediated mainly by two classes of nuclear receptors: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (Zhang and Pfahl, 1993; Kastner *et al.*, 1995; Mangelsdorf and Evans, 1995). RARs and RXRs are members of the steroid/thyroid hormone receptor superfamily that also includes a number of orphan receptors whose ligands and function remain to be determined. They modulate the expression of their target genes by interacting as either homodimers or heterodimers with RA response elements (RAREs). Some of the target genes are RARs themselves, including the RAR $\beta$  gene where a RARE ( $\beta$ RARE) was identified in its promoter region, which mediates up-regulation of RAR $\beta$  by RA in many different cell types (de The *et al.*, 1990; Hoffmann *et al.*, 1990; Sucov *et al.*, 1990).

Although the expression of RARs and RXRs is essential for the RA response, we and others recently have demonstrated that it is not sufficient to render RA target genes responsive to RA (van der Leede *et al.*, 1993; Zhang *et al.*, 1994; Kim *et al.*, 1995). In lung cancer cell lines, RARs and RXRs are well expressed, but the majority of the cell lines are RA resistant, and RA-responsive genes, such as the RAR $\beta$  gene, could not be induced by RA (Zhang *et al.*, 1994). The loss of RAR $\beta$  inducibility by RA is particularly interesting since we have observed recently that up-regulation of the RAR $\beta$  gene by RA correlates with RA-induced growth inhibition in breast cancer cell lines (Liu *et al.*, 1996) and in lung cancer cell lines (Zhang *et al.*, 1996). In RA-sensitive cancer cell lines, expression of RAR $\beta$  is strongly induced by RA. In contrast, RA had little effect on RAR $\beta$  expression in RA-resistant cancer cell lines. *In vivo*, up-regulation of RAR $\beta$  is associated with clinical response in patients with premalignant oral lesions (Lotan *et al.*, 1995). In the course of investigating the mechanism by which RAR $\beta$  is not induced by RA, we found that the expression of transfected RARs and/or RXRs could not restore RA responsiveness of the  $\beta$ RARE in certain lung cancer cell lines (Zhang *et al.*, 1994). These observations suggest that sensitivity to RA of lung cancer cells is also influenced by factors other than RARs and RXRs. One of the factors known to regulate the RA response is COUP-TF. COUP-TF is encoded by two distinct genes, COUP-TFI (ear-3) (Miyajima *et al.*, 1988; Wang *et al.*, 1989) and COUP-TFII (ARP-1) (Ladias and Karathanasis, 1991), that are orphan members of the nuclear receptor superfamily (Zhang and Pfahl, 1993; Kastner *et al.*, 1995; Mangelsdorf and Evans, 1995). Several studies recently have demonstrated that COUP-TF can repress transcription induced by a number of nuclear receptors including RARs, thyroid hormone receptors (TRs) and vitamin D receptor (VDR)

(Cooney *et al.*, 1992; Kliewer *et al.*, 1992; Tran *et al.*, 1992; Widom *et al.*, 1992), probably due to its competition for DNA binding of the receptors. The binding specificity of COUP-TFs exhibits a strong preference for those bound by retinoid receptors, suggesting that COUP-TFs are probably involved in the regulation of RA target genes.

Nur77 (also known as NGFI-B and TR3) (Chang and Kokontis, 1988; Hazel *et al.*, 1988; Milbrandt, 1988) is another orphan member of the nuclear receptor superfamily. It is induced rapidly by a variety of growth stimuli, including growth factors and phorbol esters (Hazel *et al.*, 1988; Milbrandt, 1988; Williams and Lau, 1993; Lim *et al.*, 1995). How nur77 functions to mediate the growth signaling remains largely unknown. Nur77 binds to its recognition element (NBRE) as a monomer (Wilson *et al.*, 1991). The NBRE consists of the half-site binding motif (AGGTCA) of RAR/TR/VDR with two additional adenine nucleotides at its 5' end (AAAGGTCA) (Wilson *et al.*, 1991). Interestingly, such sequences are found in the RAR $\beta$  gene promoter and are located within the  $\beta$ RARE (Perlmann and Jansson, 1995). Investigation of the binding of nur77 on the  $\beta$ RARE demonstrates that nur77 can bind to the  $\beta$ RARE as a heterodimer with RXR (Forman *et al.*, 1995; Perlmann and Jansson, 1995). These observations suggest that nur77 may be involved in the regulation of RAR $\beta$  gene expression and may function to mediate the interaction between retinoid and growth signalings (reviewed in Leblanc and Stunnenberg, 1995).

In the course of investigating the effect of nur77 on RA-induced RAR $\beta$  gene expression, we found that nur77 could significantly enhance the transactivation activity of RAREs in a RA and RARE binding-independent manner. By using a variety of approaches, we demonstrate that the effect of nur77 is due to inhibition of COUP-TF RARE binding through direct protein–protein interaction. Transient transfection analysis reveals that COUP-TF RARE binding functions to sensitize the RA responsiveness of RAREs and, conversely, that nur77 desensitizes RAREs through its ability to inhibit COUP-TF RARE binding. In human lung cancer cell lines, loss of RA sensitivity is associated with overexpression of nur77 and/or low expression levels of COUP-TF, and can be restored by introduction and expression of COUP-TF. These results reveal a novel regulatory mechanism established through heterodimerization of orphan receptors nur77 and COUP-TF, that is expected to play an important role in the regulation of retinoid sensitivity of lung cancer cells, and in the cross-talk between growth factors and vitamin A signal transduction pathways in the cells.

## Results

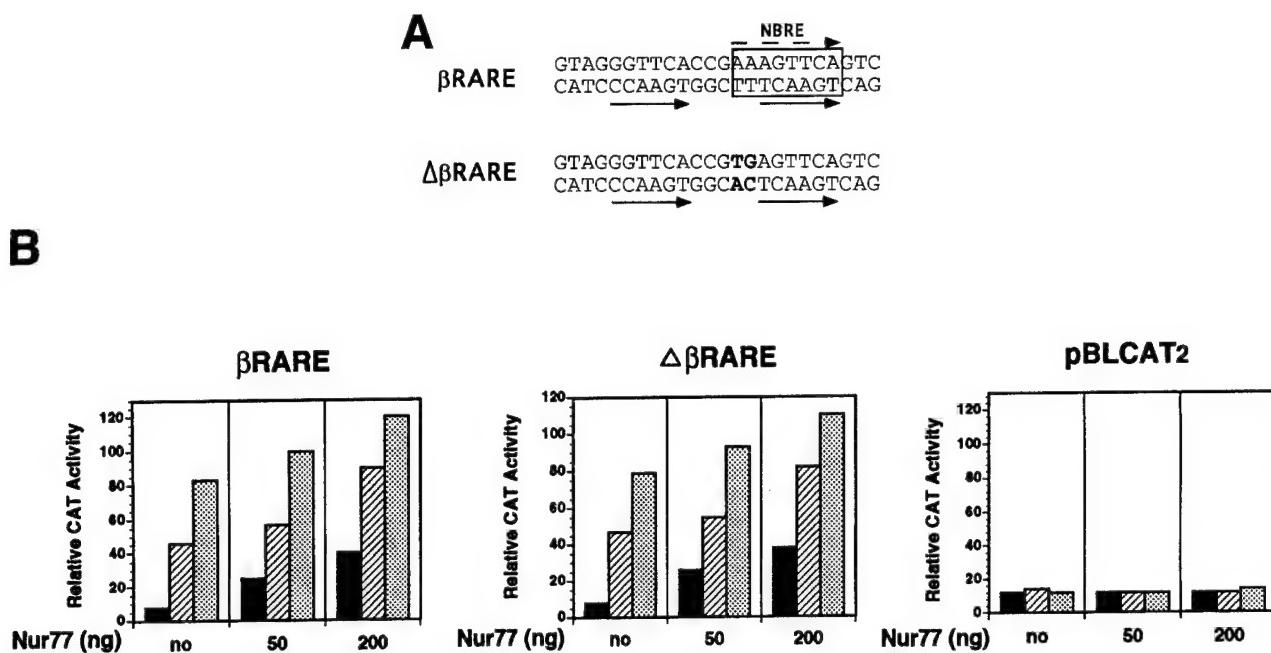
### **Nur77 enhances RARE activity in an RA-independent manner**

We have shown recently that induction of RAR $\beta$  by RA mediates the growth inhibitory effects of retinoids in human breast cancer and lung cancer cells (Liu *et al.*, 1996; Zhang *et al.*, 1996). RA-induced RAR $\beta$  expression is mediated mainly by the  $\beta$ RARE in its promoter. To investigate the effect of nur77 on the transactivation of the  $\beta$ RARE, the  $\beta$ RARE-tk-CAT that contains the  $\beta$ RARE cloned into pBLCAT<sub>2</sub> (Hoffmann *et al.*, 1990) was used as a reporter gene and was transiently transfected into CV-1 cells. When

nur77 expression vector was co-transfected, both all-*trans* RA- and 9-*cis* RA-induced reporter gene activities were enhanced in a concentration-dependent manner (Figure 1). Co-transfection of 200 ng of nur77 expression vector resulted in an ~2-fold increase of the reporter activity when cells were treated with all-*trans* RA. Surprisingly, the basal transcription of the reporter was even greatly increased, with ~5-fold enhancement. The effect of nur77 was specific to the  $\beta$ RARE because addition of nur77 did not show any activity on the parental pBLCAT<sub>2</sub> reporter. To investigate the possibility that the nur77 response is due to the presence of an NBRE within the  $\beta$ RARE, we changed two adenines in the spacing region of the  $\beta$ RARE to mutate the NBRE (Figure 1A). The mutations introduced do not affect the consensus half-site binding motifs of RAR/RXR heterodimers. The resulting element ( $\Delta\beta$ RARE) was cloned into pBLCAT<sub>2</sub> and used as a reporter. When the reporter was analyzed, we observed a similar increase in its basal transcription by co-transfection of nur77 expression vector (Figure 1). This observation suggests that the presence of an NBRE in the  $\beta$ RARE is not essential for the enhancing effect of nur77. To determine whether the enhancing effects of nur77 could be extended to other hormone response elements, reporter constructs containing the tk promoter linked to either TREpal, ApoAI-RARE, CRBPI-RARE, lactoferrin-RARE, CRBPII-RARE, a thyroid hormone-specific response element (MHC-TRE) or a CAT reporter containing the RAR $\beta$  gene promoter from -60 to +70, including the  $\beta$ RARE (Hoffmann *et al.*, 1990), were transfected into CV-1 cells with or without nur77 expression vector. Similarly to the effect on the  $\beta$ RARE, various degrees of enhancement by nur77 were observed with all the reporter constructs except the reporter containing MHC-TRE (Table I), suggesting that the effect of nur77 may be specific to RAREs. Thus, nur77 can enhance the transactivation of various RAREs in an RA-independent manner.

### **The effect of nur77 on RAREs does not require direct nur77-RARE interaction**

To investigate whether the enhancement of RARE activity by nur77 is due to its binding to the elements, gel retardation assays were performed. When the  $\beta$ RARE was used as a probe, nur77 alone did not exhibit clear binding (Figure 2A). However, a strong complex was formed when nur77 was mixed with RXR. The complex could be upshifted by anti-nur77 antibody and abolished by anti-RXR antibody, demonstrating that the complex represents RXR/nur77 heterodimers. When the  $\Delta\beta$ RARE was used as a probe, we did not see any binding of the RXR/nur77 heterodimers (Figure 2B). As a control, RXR/RAR heterodimers formed a strong complex with the element. These data indicate that the integrity of the NBRE within the  $\beta$ RARE is required for efficient RXR/nur77 binding. We also analyzed the binding of nur77 to other RAREs, such as TREpal, CRBPI-RARE, CRBPII-RARE and ApoAI-RARE, and we did not detect any binding of nur77 to these elements either in the presence or absence of RXR or RAR, except a weak RXR/nur77 heterodimer binding to the CRBPII-RARE (data not shown). Together, these results indicate that nur77 enhances the activities of different RAREs via a mechanism that is unlikely to involve a direct nur77/RARE interaction.



**Fig. 1.** RA-independent enhancement of RARE activities by nur77. (A) Sequence comparison of  $\beta$ RARE and  $\Delta\beta$ RARE. Arrows indicate receptor-binding core motifs. The nur77 binding site (NBRE) is boxed and is also indicated by the dashed arrow. Two nucleotides (in bold) of the NBRE were mutated in the  $\Delta\beta$ RARE. (B) Nur77 promotes  $\beta$ RARE and  $\Delta\beta$ RARE activities. CV-1 cells were transfected with 100 ng of the indicated CAT reporter gene together with the indicated amounts of nur77 expression vector. Cells were treated with either all-trans RA (striped bar), 9-cis RA (dotted bar) or no hormone (filled bar), and 24 h later assayed for CAT activity. CAT activity was normalized for transfection efficiency to the corresponding  $\beta$ -gal activity. Data shown represent the means of three independent experiments.

**Table I.** Nur77 activities on various RAREs and TRE

Reporter	Fold induction	
	-Ligand	+Ligand
RAR $\beta$ promoter	4.5	4.1
TREpal	4.6	2.9
ApoAI-RARE	3.5	3.0
CRBPI-RARE	7.9	4.1
CRBPII-RARE	4.8	4.0
Lactoferrin-RARE	8.3	6.6
MHC-TRE	1.1	1.2

CV-1 cells were transfected with 100 ng of CAT reporter genes containing the indicated RARE or TRE together with 200 ng of nur77 expression vector. Cells were treated with either all-trans RA ( $10^{-7}$  M) (for RAREs), thyroid hormone ( $10^{-7}$  M) (for MHC-TRE) or no hormone, and 24 h later assayed for CAT activity. CAT activity was normalized for transfection efficiency to the corresponding  $\beta$ -gal activity. Fold induction represents the ratio between relative CAT activity before and after transfection of nur77.

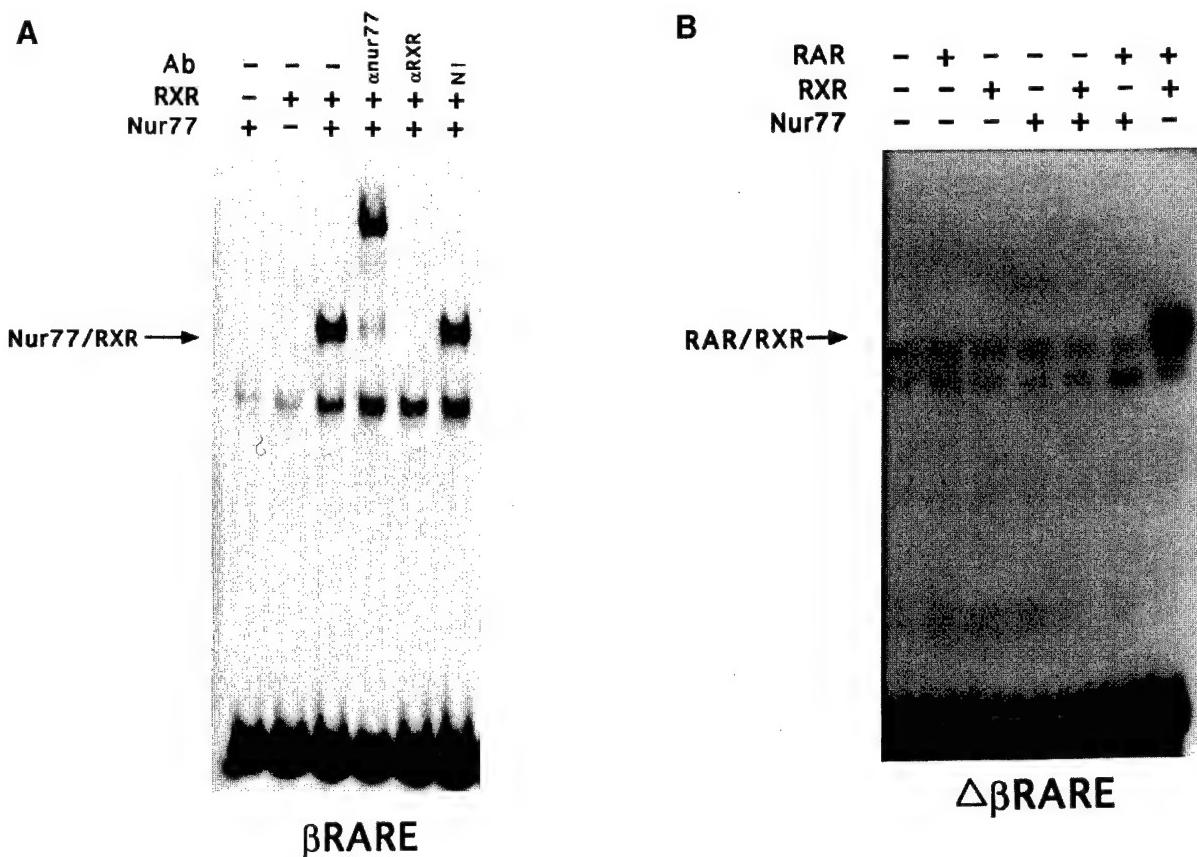
#### Nur77 inhibits COUP-TF DNA binding

The above data suggest that nur77 may function to repress the activity of an inhibitor on RAREs, thereby alleviating its inhibition. We then investigated the possibility that nur77 affects the binding of COUP-TFs that are known to bind to and restrict transcription of various RAREs (Cooney *et al.*, 1992; Kliewer *et al.*, 1992; Tran *et al.*, 1992; Widom *et al.*, 1992). We first examined the effect of nur77 on COUP-TF binding to the  $\beta$ RARE. COUP-TFI or COUP-TFII formed a strong complex with the  $\beta$ RARE (Figure 3A). However, when nur77 protein was added, the COUP-TF-RARE binding complex was inhibited. The inhibition was very efficient in that a 1 M

excess amount of nur77 significantly inhibited the COUP-TF binding and was also specific, as a similar amount of RAR had no effect on the binding (Figure 3A). This result suggests that nur77 may interact with COUP-TF, resulting in formation of nur77/COUP-TF heterodimers that cannot bind to the  $\beta$ RARE. We next investigated whether this interaction could affect nur77/RXR heterodimer binding to the  $\beta$ RARE. When COUP-TFI or COUP-TFII was incubated with nur77 and RXR, the binding of RXR/nur77 to the  $\beta$ RARE was also inhibited efficiently (Figure 3B). A 2 M excess amount of COUP-TFI or COUP-TFII was sufficient to inhibit nur77/RXR heterodimer binding. When a larger amount of COUP-TFI or COUP-TFII was used, the nur77/RXR heterodimer binding was completely inhibited and binding of COUP-TF appeared. Thus, nur77 and COUP-TF can inhibit each other's DNA binding to the  $\beta$ RARE. Nur77 could also inhibit COUP-TF binding to other RAREs, such as TREpal, CRBPI-RARE, CRBPII-RARE, and ApoAI-RARE, although the efficiency of inhibition varied among these elements (data not shown). Together, these data demonstrate that inhibition of COUP-TF DNA binding by nur77 is probably responsible for its enhancement of transactivation activity of RAREs. This is supported by our observation that COUP-TF could not bind to the MHC-TRE (data not shown) which did not show any response to nur77 (Table I).

#### Interaction of nur77 and COUP-TF in solution

To provide evidence that inhibition of COUP-TF DNA binding by nur77 on RAREs was due to a direct interaction of nur77 and COUP-TF in solution, we first performed an immunoco-precipitation assay using anti-nur77 antibody (Figure 4A). When  $^{35}$ S-labeled COUP-TFI or COUP-TFII



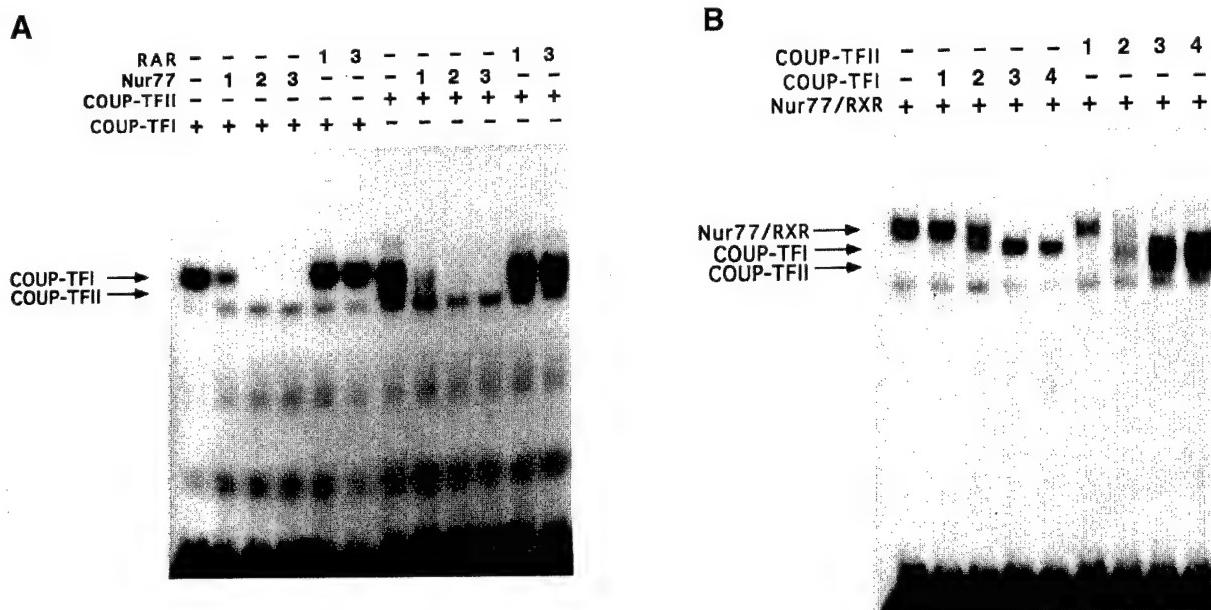
**Fig. 2.** Binding of nur77 to βRAREs. (A) Nur77 forms heterodimers with RXR on the βRARE. Equal amounts of *in vitro* synthesized nur77 and RXR were incubated alone or together at room temperature for 10 min. The reaction mixtures were then incubated with  $^{32}$ P-labeled βRARE and analyzed by gel retardation assay. When antibody (Ab) was used, it was incubated with receptor protein for 30 min at room temperature before performing the gel retardation assay. (B) Analysis of nur77 binding to ΔβRARE in the presence or absence of RAR or RXR. An equal amount of nur77 was incubated alone or together with RAR or RXR prior to performing the gel retardation assay using the indicated ΔβRARE as a probe. For comparison, the binding of RAR/RXR heterodimers is shown.

was mixed with nur77, each was precipitated by anti-nur77 antibody. The co-precipitation of COUP-TFI or COUP-TFII by anti-nur77 antibody was specific because neither could be precipitated by non-specific pre-immune serum. In addition, incubation of anti-nur77 antibody with peptide used to generate anti-nur77 antibody prevented its precipitation. To study the interaction further, we cloned nur77 cDNA into pGEX-2T expression vector and expressed a GST-nur77 fusion protein in bacteria. The fusion protein was immobilized on glutathione-Sepharose beads, and mixed with either  $^{35}$ S-labeled COUP-TFI or COUP-TFII protein. For comparison, labeled RAR $\alpha$  and RXR $\alpha$  were used. As shown in Figure 4B, the labeled COUP-TFI or COUP-TFII protein bound specifically to nur77-immobilized Sepharose beads but not to the control beads, demonstrating the specific interaction between nur77 and COUP-TF in solution. Under the conditions used, we did not observe a clear binding of RAR $\alpha$  or RXR $\alpha$  to the nur77-immobilized Sepharose beads, suggesting that interaction between nur77 and COUP-TF in solution is much stronger than nur77-RXR interaction. To study whether nur77 and COUP-TF could interact *in vivo*, we cloned nur77 in-frame into the yeast expression vector pGAD424 that contains the Gal4 activation domain, and COUP-TF into the yeast expression vectors pGBT9 that contains the Gal4 DNA-binding domain (DBD). The

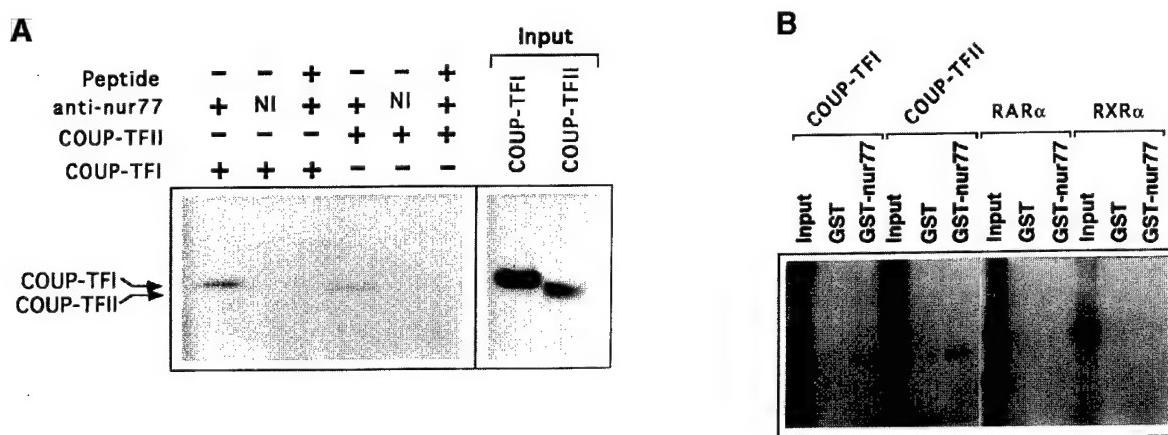
resulting vectors, pGBT9/nur77 and pGAD424/COUP-TF, were analyzed for their interaction *in vivo* by the yeast two-hybrid system (Bartel *et al.*, 1993). Transformation of either COUP-TF and empty vector pGAD424, or nur77 and empty vector pGBT9 into Y190 yeast cells could not activate the LacZ reporter containing the Gal4-binding site. However, when COUP-TF was transformed together with nur77 the reporter was strongly activated (Figure 5). Thus, nur77 and COUP-TF can interact in intact cells. Together, these data demonstrate that nur77 can inhibit COUP-TF DNA binding through a direct protein-protein interaction.

#### Nur77 regions required for interaction with COUP-TF

Nuclear receptors are characterized by a highly conserved DBD, a well conserved ligand-binding domain (LBD) located at the carboxy-terminal half of the receptor. In addition to ligand binding, the C-terminal region is critical in receptor homo- and heterodimerization (Zhang and Pfahl, 1993; Kastner *et al.*, 1995; Mangelsdorf and Evans, 1995). To determine whether a similar domain of nur77 is employed in the interaction with COUP-TF, two deletion mutants of nur77, Δnur77-1 and Δnur77-2, were constructed (Figure 6A) and tested for their interaction with COUP-TF by the yeast two-hybrid assay (Figure 6B). In Δnur77-1, a segment



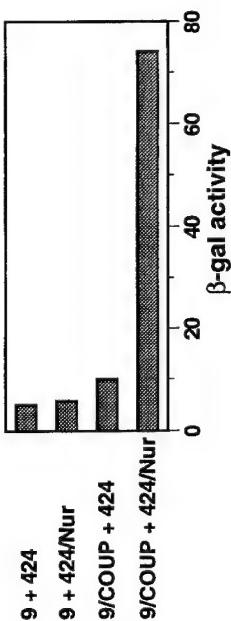
**Fig. 3.** Mutual inhibition of nur77 and COUP-TF DNA binding. (A) Inhibition of COUP-TF DNA binding on the  $\beta$ RARE by nur77. *In vitro* synthesized COUP-TF was pre-incubated with the indicated molar excess of nur77. Unprogrammed reticulocyte lysate was used to maintain an equal protein concentration in each reaction. Following this pre-incubation, the reaction mixtures were incubated with  $^{32}$ P-labeled  $\beta$ RARE and analyzed by the gel retardation assay. For the control, COUP-TF was also pre-incubated with the indicated molar excess of *in vitro* synthesized RAR $\alpha$  protein. (B) Inhibition of nur77/RXR heterodimer binding on the  $\beta$ RARE by COUP-TFs. *In vitro* synthesized nur77 protein was pre-incubated with RXR $\alpha$  in the presence or absence of the indicated molar excess of COUP-TFI or COUP-TFII, and analyzed by gel retardation assay using the  $\beta$ RARE as a probe. Unprogrammed reticulocyte lysate was used to maintain an equal protein concentration in each reaction.



**Fig. 4.** Direct interaction of nur77 and COUP-TF in solution. (A) Analysis of nur77-COUP-TF interaction by the immunoprecipitation assay.  $^{35}$ S-Labeled *in vitro* synthesized COUP-TFI or COUP-TFII was incubated with *in vitro* synthesized nur77. After incubation, either anti-nur77 antibody or non-specific pre-immune serum (NI) was added. In the control, anti-nur77 antibody was pre-incubated with a peptide from which the antibody was generated. The immune complexes were washed, boiled in SDS sample buffer and separated on a 10% SDS-PAGE. The inputs of the labeled COUP-TFI and COUP-TFII are shown for comparison. (B) Analysis of nur77-COUP-TF interaction by the GST pull down assay. To analyze the interaction between nur77 and COUP-TFs further, nur77 protein was synthesized in bacteria using the pGEX-2T expression vector (Pharmacia). The GST/nur77 fusion protein was immobilized on glutathione-Sepharose beads. As a control, the same amount of GST was also immobilized on the beads.  $^{35}$ S-Labeled COUP-TFI, COUP-TFII, RAR $\alpha$  or RXR $\alpha$  was then mixed with the beads. After extensive washing, the bound proteins were analyzed by SDS-PAGE. The input proteins are shown for comparison.

of amino acids from 168 to 468 was removed, whereas in  $\Delta$ nur77-2, 126 amino acids were deleted from the C-terminal end of nur77. Both mutants were cloned in-frame into pGAD424. The resulting vectors, pGAD424- $\Delta$ nur77-1 and pGAD424- $\Delta$ nur77-2, were analyzed for their interaction with COUP-TF. Co-expression of  $\Delta$ nur77-1 and

COUP-TF did not show any activation of the reporter, suggesting that the deleted region is required for nur77 to interact with COUP-TF. Unexpectedly, the expression of  $\Delta$ nur77-2 together with COUP-TF strongly activated the reporter to a degree similar to that observed with wild-type nur77. Further deletion of 28 amino acids from the



**Fig. 5.** Nur77 interacts with COUP-TF in yeast. The *nur77* and COUP-TF cDNAs were cloned into the yeast expression vectors pGAD424 (424) and pGBT9 (9), respectively. The resulting expression vectors, 424/nur and 9/COUP, were introduced into Y190 yeast cells as indicated.  $\beta$ -Galactosidase activity was assayed from a yeast strain Y190 containing the LacZ reporter plasmid to study the *in vivo* interaction. The  $\beta$ -galactosidase activity measured with the indicated combinations of yeast expression vectors is shown for comparison.

C-terminal end of  $\Delta$ nur77-2 did not affect its interaction with COUP-TF (data not shown). These data demonstrate that a putative domain is utilized by nur77 to interact with COUP-TF. We also analyzed the domain requirement of COUP-TF. In contrast to nur77, deletion of a region encompassing the DBD ( $\Delta$ COUP-TF-1) or 116 amino acids from the C-terminal end ( $\Delta$ COUP-TF-2) completely abolished its interaction with nur77, suggesting that both regions are required.

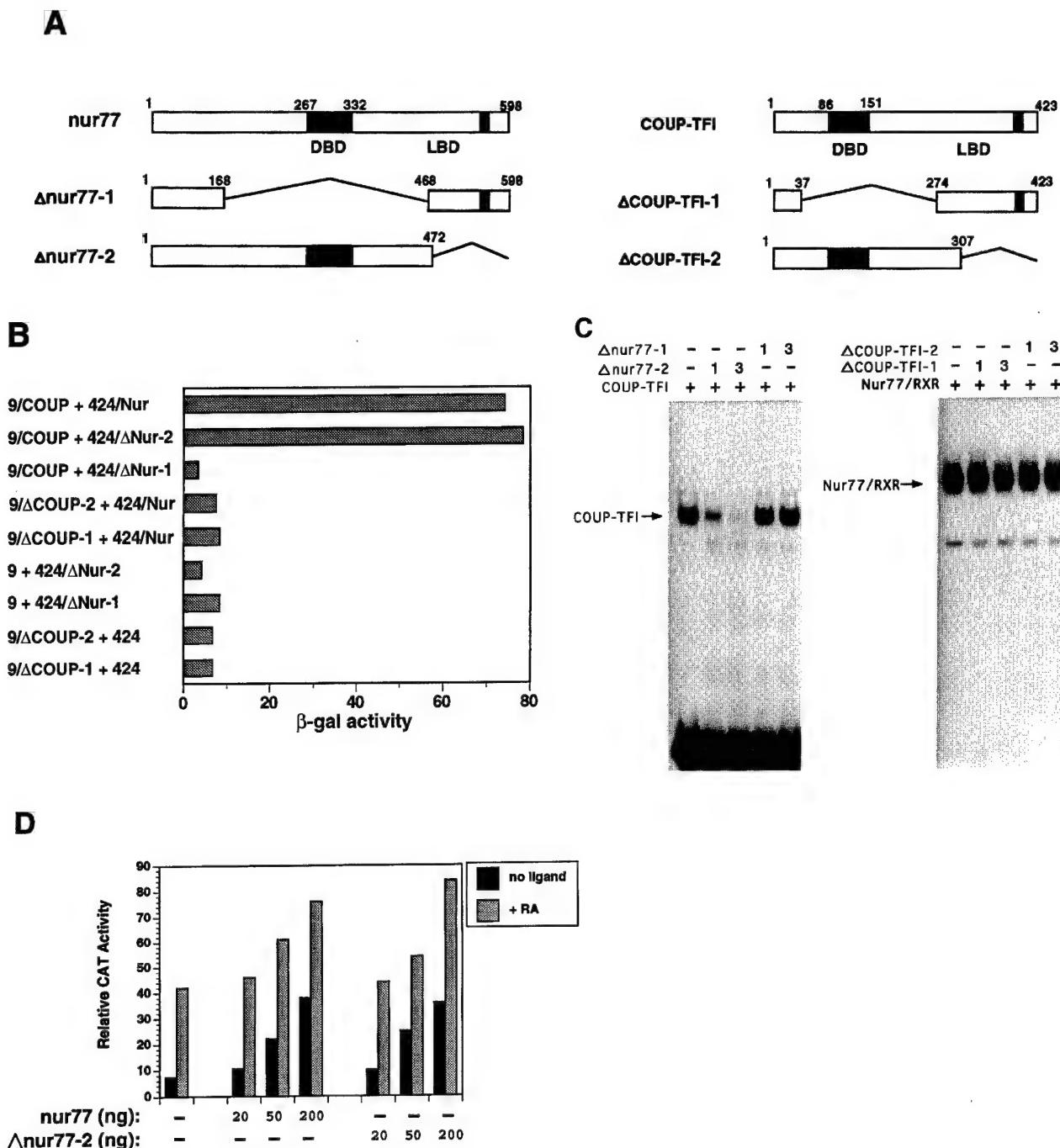
To investigate the interaction of the mutants further, a gel retardation assay was conducted by using the  $\beta$ RARE as a probe (Figure 6C). Similarly to what was observed with wild-type nur77 (Figure 3A),  $\Delta$ nur77-2 effectively inhibited the binding of COUP-TF to the  $\beta$ RARE. In contrast,  $\Delta$ nur77-1 did not show any effect. When the effect of COUP-TF mutants on nur77/RXR heterodimer binding was analyzed, we did not observe any inhibition of nur77/RXR binding. To study whether  $\Delta$ nur77-2 retained its ability to enhance the basal activity of RAREs,  $\Delta$ nur77-2 cloned into the pCEP expression vector was transfected into CV-1 cells together with  $\beta$ RARE-tk-CAT (Figure 6D). Like wild-type nur77,  $\Delta$ nur77-2 could also enhance the reporter activity in a RA-independent manner. Similar results were obtained when reporters containing other RAREs were used (data not shown). These gel retardation and transfection data are consistent with the yeast two-hybrid results and suggest that the interaction between nur77 and COUP-TF is mediated by a mechanism that is different from that employed by many other nuclear receptors.

#### **Antagonistic effect of *nur77* and COUP-TF on modulating the RA sensitivity of RAREs**

The previous demonstration that COUP-TF can inhibit RA-induced activity was based mainly on transient co-transfection assays where COUP-TF might be over-expressed (Cooney *et al.*, 1992; Kliewer *et al.*, 1992; Tran *et al.*, 1992; Widom *et al.*, 1992). We then examined the effect of various concentrations of COUP-TF on the  $\beta$ RARE activity. Co-transfection of larger amounts of COUP-TF expression vector with the  $\beta$ RARE-tk-CAT reporter almost completely inhibited RA-induced reporter activity, consistent with previous results (Cooney *et al.*, 1992; Kliewer *et al.*, 1992; Tran *et al.*, 1992; Widom *et al.*, 1992). However, at low concentrations (1, 5 or 10 ng), COUP-TF either did not affect or even slightly enhanced the RA-induced  $\beta$ RARE activity (Figure 7A). At these concentrations, COUP-TF significantly inhibited the basal activity of the reporter, resulting in an increase of RA-dependent fold induction of the  $\beta$ RARE activity (Figure 7B). Without co-transfection of COUP-TF, a 4-fold induction by RA was seen. However, when 10 ng of COUP-TF expression vector was co-transfected, we observed a 14-fold induction of reporter activity in response to RA. These data are in agreement with observations made previously on the peroxisome proliferator responsive element (PPRE) (Baes *et al.*, 1995) and ApoAI-RARE (Widom *et al.*, 1992), where co-transfection of COUP-TF enhanced the hormonal sensitivity of both responsive elements. Thus, COUP-TF, at appropriate concentrations that are likely to occur in most cells, can enhance the RA sensitivity of the  $\beta$ RARE. To analyze the effect of *nur77* on COUP-TF activity, we co-transfected *nur77* expression vector together with COUP-TF. As shown in Figure 7A, the inhibition of basal transcription of the  $\beta$ RARE by COUP-TF was alleviated completely when *nur77* was co-transfected, resulting in a decrease in RA-dependent fold induction of the  $\beta$ RARE activity (Figure 7B). These data, together with the results shown in Figure 1, demonstrate that *nur77* can desensitize the RA responsiveness of RAREs by antagonizing COUP-TF transcriptional activity.

#### **Expression of *nur77* and COUP-TF in human lung cancer cell lines**

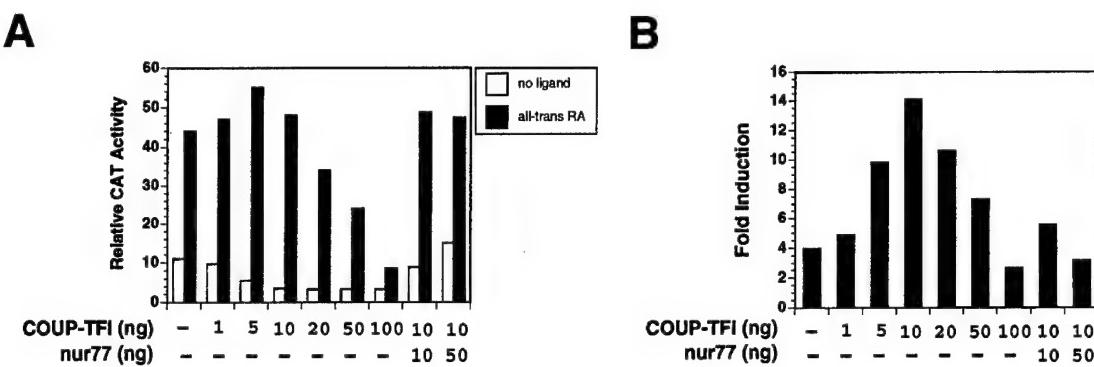
The above observations prompted us to investigate whether expression levels of *nur77* and COUP-TF correlate with RA sensitivities observed in various lung cancer cell lines (Figure 7). These cancer cell lines displayed various degrees of RA sensitivity in inducing  $\beta$ RARE despite the fact that RARs and RXRs are well expressed (Zhang *et al.*, 1994; data not shown). Although the degree of RA induction of  $\beta$ RARE activity may also depend on levels of retinoid receptors expressed in the cell lines, we found a perfect correlation between COUP-TF expression and RA induction of  $\beta$ RARE activity (Figure 8). COUP-TF was well expressed in Calu-6, H460, H596, SK-MES-1 and H661 lung cancer cell lines, in which  $\beta$ RARE activity was highly induced by RA. In contrast, COUP-TF transcripts were not detected in other cancer cell lines in which the  $\beta$ RARE was not induced by RA. These observations suggest that the expression of COUP-TF in these cancer cell lines does not repress RA-induced transactivation activity but is required for RA-dependent transactivation



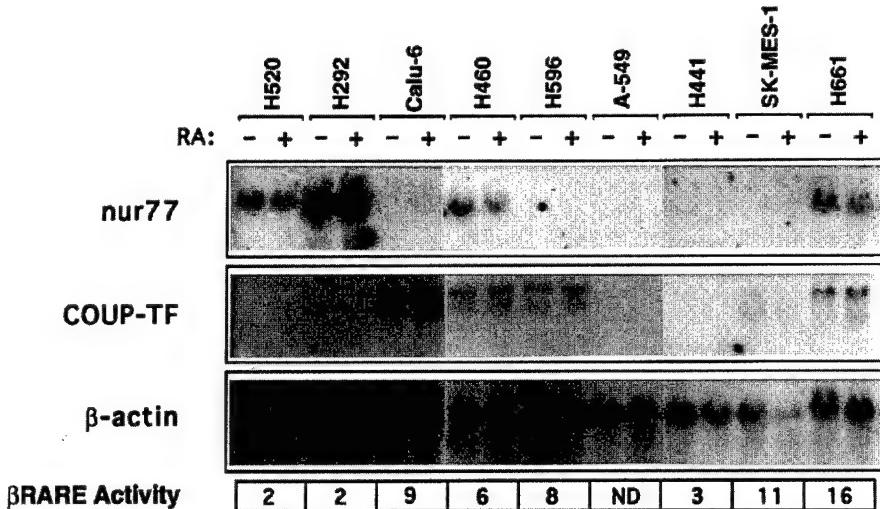
**Fig. 6.** Domain requirements for *nur77*-COUP-TF interaction. (A) Schematic representation of the *nur77* and COUP-TF deletion mutants. The DNA-binding domain (DBD) and ligand-binding domain (LBD) are indicated. Amino acid numbers are indicated above the bar. (B) *Δnur77-2* interacts with COUP-TF in yeast. *Δnur77-1* and *Δnur77-2* were cloned into the pGBT9 vector in-frame and *ΔCOUP-TF-1* and *ΔCOUP-TF-2* were cloned into pGBT9 in-frame. The resulting expression vectors, 424/*Δnur-1*, 424/*Δnur-2*, 9/*ΔCOUP-1* and 9/*ΔCOUP-2*, were introduced into yeast Y190 cells as indicated.  $\beta$ -Gal activity was measured in yeast cells. For comparison, the interaction between wild-type *nur77* and COUP-TF is shown. (C) *Δnur77-2* inhibits COUP-TF DNA binding. To investigate the interaction between *nur77* and COUP-TF further, *nur77* deletion mutants were synthesized by *in vivo* transcription-translation, and analyzed for their effect on COUP-TF binding to the  $\beta$ RARE by gel retardation. Similarly, the effect of COUP-TF1 deletion mutants on *nur77/RXR* binding was analyzed. (D) *Δnur77-2* enhances  $\beta$ RARE activity. CV-1 cells were transfected with 100 ng of  $\beta$ RARE-tk-CAT together with the indicated amounts of *Δnur77-2* expression vector. Cells were treated with or without  $10^{-7}$  all-trans RA and assayed for CAT activity. The effect of the wild-type *nur77* is shown for comparison.

of the  $\beta$ RARE. Hence, COUP-TF may sensitize  $\beta$ RARE responsiveness to RA through its binding to the element. When the expression of *nur77* was analyzed, we found that it was highly expressed in RA-resistant H520 and H292 lung cancer cell lines. Although high levels of *nur77*

were also observed in RA-sensitive H661 and H460 cell lines, these cell lines expressed significant amounts of COUP-TF, that may counteract the effect of *nur77*. Under the conditions used, we did not detect expression of *nur77* in the RA-resistant H441 cell line. It is likely that factors



**Fig. 7.** Modulation of RA sensitivity of  $\beta$ RARE by COUP-TF and nur77. (A)  $\beta$ RARE-tk-CAT was co-transfected with the indicated amounts of nur77 and/or COUP-TF into CV-1 cells. Cells were treated with or without  $10^{-7}$  M all-trans RA, and 24 h later assayed for CAT activity. Data shown represent the means of three independent experiments. (B) The same data were plotted to indicate the fold activation by RA.



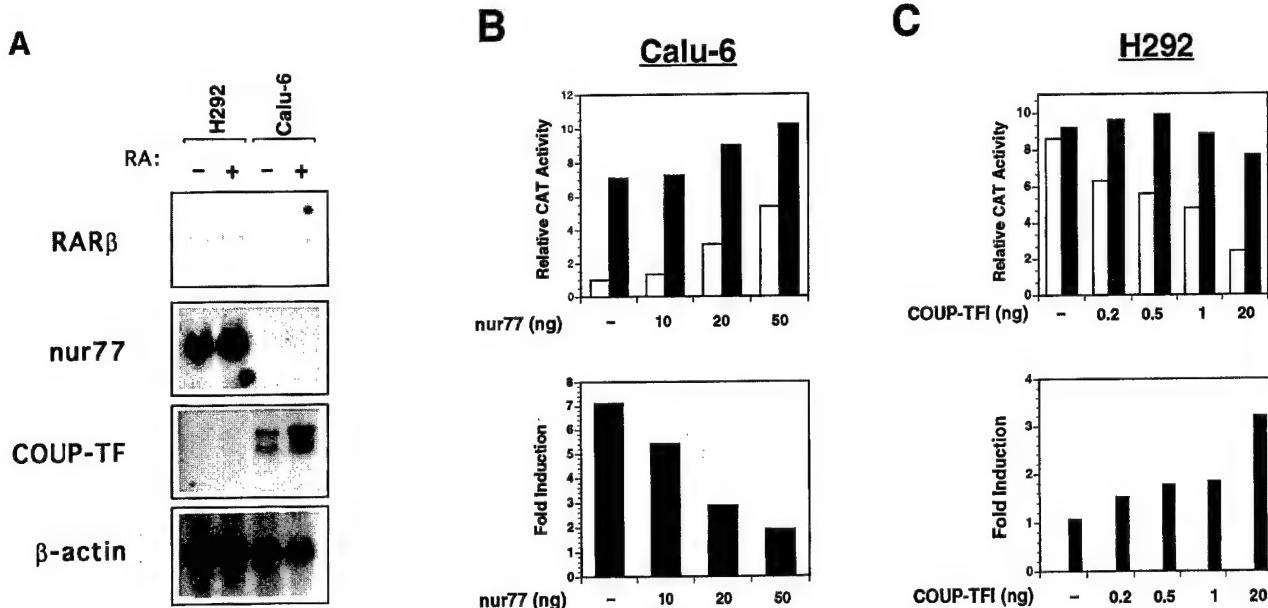
**Fig. 8.** Expression of COUP-TF and nur77 and RA-dependent  $\beta$ RARE activity in human lung cancer cell lines. Total RNAs were prepared from the indicated human lung cancer cell lines treated with or without  $10^{-6}$  M all-trans RA for 24 h and analyzed for the expression of COUP-TF and nur77. As a control, the expression of  $\beta$ -actin is shown.  $\beta$ RARE activity represents the fold induction by all-trans RA as determined by transient transfection assay using the  $\beta$ RARE-tk-CAT as a reporter.

other than nur77 may be responsible for RA resistance in these cells.

#### Dynamic balance of nur77 and COUP-TF regulates RA sensitivity in human lung cancer cell lines

In our previous studies, we observed that RAR $\beta$  was differentially expressed in several human lung cancer cell lines (Zhang *et al.*, 1994). RAR $\beta$  was not expressed in Calu-6 lung cancer cells but its expression was greatly induced by RA treatment. In contrast, RAR $\beta$  was highly expressed in H292 lung cancer cells but in an RA-independent manner (Figure 9A). COUP-TF is expressed in RA-sensitive Calu-6 but not in RA-resistant H292 cells, whereas nur77 is expressed in H292 but not in Calu-6 cells. This suggests that relative expression levels of COUP-TF and nur77 may affect expression of the RAR $\beta$  gene. We therefore analyzed whether co-transfection of nur77 or COUP-TF affects the RA sensitivity of the  $\beta$ RARE in Calu-6 and H292 cells. We first investigated the effect of nur77 in RA-sensitive Calu-6 cells (Figure 9B). When nur77 expression vector was co-transfected together with the  $\beta$ RARE-tk-CAT into the cells, we observed an increase in basal activity

and a decrease in RA-dependent fold induction of the reporter. Co-transfection of 50 ng of nur77 expression vector reduced RA-dependent  $\beta$ RARE activity from 7-fold to 2-fold. This result suggests that the high sensitivity of Calu-6 cells to RA may be due to a low expression level of nur77. We next analyzed the effect of COUP-TF on RA-resistant H292 cells (Figure 9C). Co-transfection of COUP-TF expression vector with the  $\beta$ RARE-tk-CAT into the cells reduced basal reporter activity while RA-induced activity was not clearly affected. In the absence of COUP-TF, we did not see a clear effect of RA on  $\beta$ RARE activity. However, when 20 ng of COUP-TF expression vector was co-transfected, we found a 3-fold induction of the  $\beta$ RARE activity by RA. This data demonstrates that loss of RA sensitivity in H292 cells may be due to a low level of COUP-TF in the cells. In addition, nur77 expressed in H292 cells may further inhibit the COUP-TF effect. Thus, a dynamic balance of nur77 and COUP-TF is important in regulating the RA sensitivity of the  $\beta$ RARE in these cancer cells and overexpression of nur77 and/or lack of COUP-TF may be responsible for RA resistance in H292 cells.



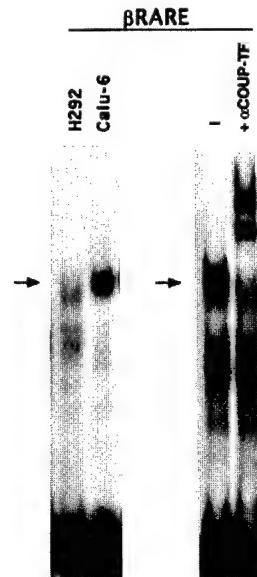
**Fig. 9.** Modulation of RA sensitivity by COUP-TF and nur77 in human lung cancer cell lines. (A) Effect of RA in inducing RAR $\beta$  expression in Calu-6 and H292 cell lines. Total RNAs were prepared from Calu-6 or H292 lung cancer cells treated with or without  $10^{-6}$  M all-trans RA for 24 h and analyzed for the expression of RAR $\beta$ . For comparison, expression of nur77 and COUP-TF is shown. The expression of  $\beta$ -actin is used as a control. (B) Nur77 decreases RA sensitivity in Calu-6 cells.  $\beta$ RARE-tk-CAT was co-transfected with the indicated amounts of COUP-TFI into Calu-6 cells. The cells were treated with (filled bars) or without (empty bars)  $10^{-7}$  M all-trans RA for 24 h, and assayed for CAT activity. Data shown represent the means of two experiments. (C) COUP-TF enhances RA sensitivity in H292 cells,  $\beta$ RARE-tk-CAT was co-transfected with the indicated amounts of COUP-TFI into H292 cells. The cells were then treated with (filled bars) or without (empty bars)  $10^{-7}$  M all-trans RA for 24 h and assayed for CAT activity. Data shown are representative of four independent experiments.

#### **COUP-TF or COUP-TF-like protein forms a major complex with $\beta$ RARE in RA-resistant lung cancer cells**

The above data suggest that COUP-TF may enhance RA sensitivity through its binding to RAREs. To test directly that COUP-TF expressed in RA-sensitive Calu-6 lung cancer cells binds to RARE, nuclear proteins were prepared from Calu-6 cells and RA-resistant H292 cells and analyzed for their RARE binding by gel retardation using the  $\beta$ RARE as a probe. As shown in Figure 10, in addition to several weak complexes, a strong  $\beta$ RARE-binding complex (indicated by the arrow) was observed with nuclear proteins from Calu-6 but not from H292 cells. To determine whether COUP-TF contributed to the  $\beta$ RARE binding, nuclear proteins from Calu-6 cells were incubated with anti-COUP-TF antibody prior to the gel retardation assay. Interestingly, the major  $\beta$ RARE-binding complex was completely upshifted by the anti-COUP-TF antibody, while binding of other weak binding complexes was not affected. Similar results were obtained when CRBPI-RARE was used as a probe (data not shown). Thus, these data clearly demonstrate that the binding of COUP-TF to the  $\beta$ RARE contributes to its effect on the RA sensitivity of the RARE in these lung cancer cell lines.

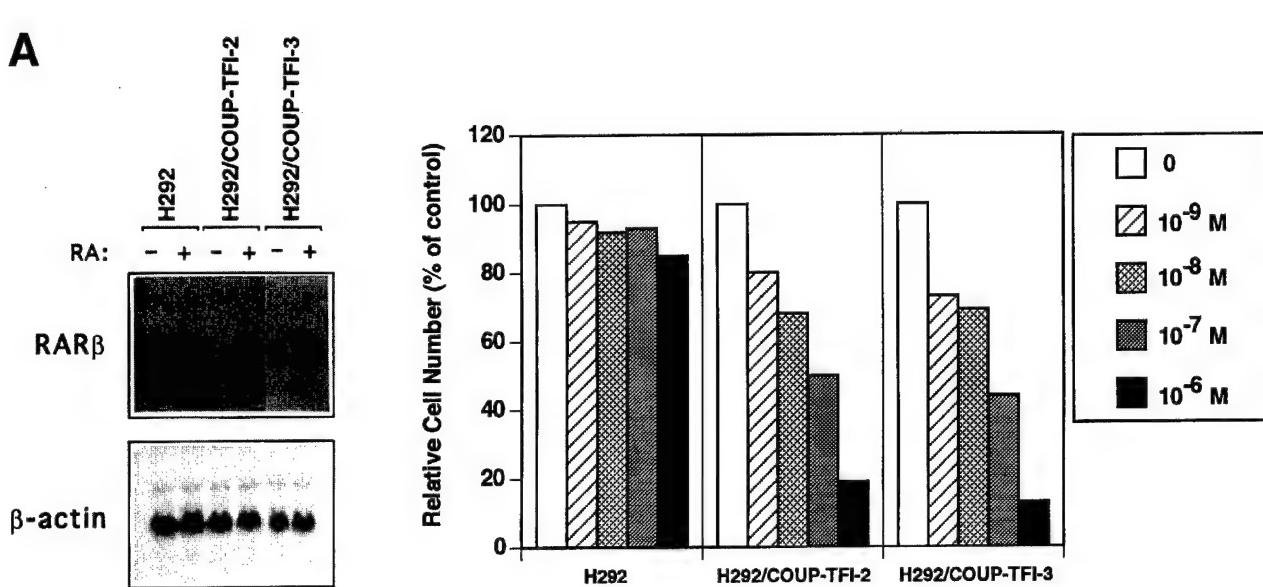
#### **Stable expression of COUP-TF restores RA sensitivity in RA-resistant human lung cancer cells**

The observations that nur77 and COUP-TF are differentially expressed in RA-sensitive Calu-6 and RA-resistant H292 cells (Figure 8) and that they can antagonize each other's transcriptional activity (Figure 9) suggest



**Fig. 10.** COUP-TF or COUP-TF-like protein contributes to the  $\beta$ RARE binding activity in an RA-sensitive, COUP-TF-positive lung cancer cell line. Nuclear proteins were prepared from COUP-TF-positive Calu-6 and -negative H292 cells, and analyzed for their DNA binding activity using  $\beta$ RARE as a probe. Nuclear proteins from Calu-6 cells were also analyzed for the effect of anti-COUP-TF antibody. The arrow indicates the binding complex(es) present in Calu-6 but not in H292 cells.

the possibility that constitutive expression of RAR $\beta$  in H292 cells may be due to overexpression of nur77 and lack of COUP-TF in the cells. To test whether expression of COUP-TF could antagonize the effect of nur77 and



**Fig. 11.** Stable expression of COUP-TF in RA-resistant H292 cells restores their RA sensitivity. (A) Expression of the RAR $\beta$  gene in H292 and stable clones. Total RNAs were prepared from Calu-6 and H292 human lung cancer cell lines treated with or without  $10^{-6} M$  all-trans RA for 24 h and analyzed for the expression of RAR $\beta$ . In the control, the expression of  $\beta$ -actin is shown. (B) RA-induced growth inhibition in H292 and H292 stable clones that expressed transfected COUP-TF. Cells were seeded at 1000 cells per well in a 96-well plate and treated with the indicated concentrations of all-trans RA for 6 days. The numbers of viable cells were determined by the MTT assay.

sensitize RAR $\beta$  expression responsiveness to RA in H292 cells, we stably expressed COUP-TF in the cells. Two stable clones (H292/COUP-TFI-2 and H292/COUP-TFI-3) that expressed COUP-TF were subjected to analysis of their RAR $\beta$  gene expression in the absence or presence of RA. In the absence of RA, the level of RAR $\beta$  expression in these stable clones was largely reduced (Figure 11A), consistent with our transient transfection results (Figure 9C). Surprisingly, when the stable clones were exposed to RA, the reduced level of RAR $\beta$  was significantly enhanced to the level observed in parental H292 cells (Figure 11A). This, again, is consistent with our transient transfection data (Figure 9C), and provides strong evidence that appropriate levels of COUP-TF expression do not inhibit RA-induced  $\beta$ RARE activity. RA did not significantly inhibit the growth of parental H292 cells (Figure 11B). However, it could now strongly inhibit the growth of the stable clones, with  $\sim 85\%$  inhibition observed when the cells were treated with  $10^{-6} M$  RA for 6 days (Figure 11B). Thus, the expression of COUP-TF could sensitize RA responsiveness of RAR $\beta$  expression and growth inhibition in RA-resistant H292 lung cancer cells by reducing the basal activity of the  $\beta$ RARE in the absence of RA.

## Discussion

The diverse functions of RA are mediated mainly by RARs and RXRs. However, expression of RARs and RXRs is often not sufficient to render cells RA responsive. Here we provide evidence that orphan receptors COUP-TF and nur77 play a critical role in the regulation of RA responsiveness of various RA target genes through their modulation of RARE binding. COUP-TFs bind to a

variety of RAREs and sensitize their RA responsiveness. Conversely, nur77 reduces RA sensitivity of RAREs through heterodimerization with COUP-TF, which results in inhibition of COUP-TF binding to RAREs. These observations reveal a novel mechanism that modulates RA responses through heterodimerization of orphan receptors COUP-TF and nur77.

### COUP-TFs function to sensitize the RA responsiveness of RAREs

Results from several previous studies demonstrate that COUP-TFs function to inhibit RA-induced transactivation of RAREs (Cooney *et al.*, 1992; Kliewer *et al.*, 1992; Tran *et al.*, 1992; Widom *et al.*, 1992). We demonstrate here that COUP-TFs may also function to sensitize the RA responsiveness of RA target genes by reducing their basal activity. In transient transfection assays in CV-1 (Figure 7) and in lung cancer cells (Figure 9), expression of appropriate amounts of COUP-TF repressed the basal transcription of the  $\beta$ RARE-tk-CAT reporter while it had no effect on RA-induced reporter activity. This results in an increase of RA sensitivity of the  $\beta$ RARE (Figures 7 and 9). In a previous study, Baes *et al.* (1995) also observed that COUP-TF inhibited the basal level of the PPRE in the absence of exogenously added ligands. These observations suggest that the sensitizing effect of COUP-TF may represent a general regulatory mechanism of COUP-TF functions. The sensitizing effect of COUP-TF on RAREs is probably due to its binding to the elements since COUP-TF binds strongly to RAREs *in vitro* (Figure 3). In addition, in RA-sensitive Calu-6 lung cancer cells, COUP-TF formed a strong complex with the  $\beta$ RARE, while such a complex was not seen in RA-resistant H292 lung cancer cells that constitutively express RAR $\beta$  (Figure 10). Hence, the binding of COUP-TF to RAREs

may prevent them from binding and activation by certain RA-independent activators, such as MB67 that binds and activates the  $\beta$ RARE in an RA-independent manner (Baes et al., 1994).

The notion that COUP-TF functions to maintain the RA sensitivity of RAREs by binding to the elements would require that the binding of COUP-TF be replaced by retinoid receptors once retinoids are available. This would suggest that retinoid receptors, upon binding to RA, gain affinity for RAREs. We demonstrated previously that binding of RXR homodimers to RAREs was promoted by its ligand 9-cis RA *in vitro* (Zhang et al., 1992b). Although RA does not show a clear effect on RAR/RXR heterodimer DNA binding *in vitro* (Zhang et al., 1992a,b), it was observed, by using *in vivo* footprinting, that RAR-RXR heterodimers do not occupy the  $\beta$ RARE in the absence of RA in P19 cells (Dey et al., 1994) and that RAR ligands can promote retinoid receptor  $\beta$ RARE binding *in vivo* (Chen et al., 1996). Thus, it is likely that, *in vivo*, the ligand induces conformational changes of retinoid receptors so that they have a higher affinity for RARE, that would allow them to replace COUP-TF RARE binding, and subsequently RA responses. Whether liganded retinoid receptors are capable of replacing COUP-TF binding on a RARE may also depend on the binding affinity of COUP-TF for the RARE and expression levels of COUP-TF. For example, COUP-TF has a relatively low affinity for the  $\beta$ RARE (Tran et al., 1992) so that the binding of COUP-TFs may be easily replaced by liganded retinoid receptors. In contrast, the affinity of COUP-TFs for some other RAREs, such as TREpal, is much higher and the binding of COUP-TF to these RAREs may not be replaced easily by retinoid receptors even though they are complexed with ligands. This may explain our observations that certain RAREs, such as TREpal, could not be activated by RA-induced endogenous receptors in RA-sensitive Calu-6 cells while  $\beta$ RARE is highly activated in the same cells (data not shown). Similarly, endogenous receptors in CV-1 cells are sufficient to activate  $\beta$ RARE but not TREpal (Zhang et al., 1992b). Hence, COUP-TF may act to sensitize certain RAREs to their RA responsiveness while at the same time functioning as a silencer of other RAREs depending on RARE binding affinity and expression levels.

#### **Nur77 modulates RARE activity through interaction with COUP-TF**

Nur77 is an immediate-early protein whose expression is induced rapidly by a variety of growth stimuli (Hazel et al., 1988; Milbrandt, 1988; Williams and Lau, 1993; Lim et al., 1995). However, the function of nur77 and its mechanism of action remain largely unknown. In the present study, we show that nur77 can enhance the transcriptional activity of a variety of RAREs in an RA-independent manner (Figure 1). Enhancement of RARE activity does not require a direct interaction of nur77 with RARE, since nur77, alone or in the presence of RAR or RXR, does not bind to RAREs except to the  $\beta$ RARE (Figure 2 and data not shown). Binding of nur77/RXR heterodimers to the  $\beta$ RARE may be an alternative mechanism to activate the  $\beta$ RARE since the heterodimers can be induced by certain RXR-selective retinoids (Forman et al., 1995; Perlmann and Jansson, 1995). Our DNA binding

experiments indicate that the effect of nur77 is mediated largely by its inhibition of COUP-TF RARE binding. A 2 M excess of nur77 almost completely inhibited COUP-TF binding on the  $\beta$ RARE when nur77 was pre-incubated with COUP-TF (Figure 3). However, if COUP-TF is pre-bound to the RARE, it becomes relative refractory to the inhibitory action of nur77 (data not shown). The inhibition of COUP-TF RARE binding activity by nur77 is likely to be mediated by direct interaction between nur77 and COUP-TF in solution, as demonstrated by our immunoprecipitation (Figure 4A) and GST pull down experiments (Figure 4B). By using the yeast two-hybrid assay, we show that the interaction can occur *in vivo* (Figure 5). In a transient transfection assay, nur77 can counteract the effect of co-transfected COUP-TF in CV-1 cells (Figure 7). These observations clearly demonstrate that nur77 exerts its effect on RAREs through interaction with COUP-TF, forming complexes that do not bind to the RAREs.

We used deletion mutants to identify domains in COUP-TF and nur77 responsible for interaction. One surprising result is that a large portion of the putative LBD of nur77 is not required for the interaction (Figure 6). This is unexpected because the C-terminal half of nuclear receptors is essential for homo- and heterodimerization of many nuclear receptors, such as RARs, RXR, T3R and VDR (Zhang and Pfahl, 1993; Kastner et al., 1995; Mangelsdorf and Evans, 1995). Our observation would then suggest that the DBD or adjacent sequences is involved in protein-protein interaction or that they are required for other domains in the receptor to achieve the proper conformation for interaction. On the other hand, the A/B region of nur77 is relatively large as compared with other nuclear receptors and may contain sequences responsible for interaction. A detailed analysis will determine the putative domain in nur77 required for interaction with COUP-TF.

#### **Regulation of retinoid sensitivity and RAR $\beta$ expression in lung cancer cells by COUP-TF and nur77**

The observation that expression of COUP-TF is required to maintain RA sensitivity and that nur77 can antagonize the effect of COUP-TF provides a framework for understanding the retinoid sensitivity in cancer cells. This becomes especially apparent since retinoid resistance is observed frequently in various types of cancer cells despite expression of functional retinoid receptors (van der Leede et al., 1993; Zhang et al., 1994; Kim et al., 1995). In human lung cancer cell lines, RARs and RXRs are well expressed, but many of the cell lines show resistance to RA-induced growth inhibition (Zhang et al., 1996) and RAR $\beta$  expression (Zhang et al., 1994). Our observation that COUP-TF expression is positively correlated with RA sensitivity in lung cancer cell lines (Figure 8) demonstrates that COUP-TF is required for RA sensitivity in the cells. COUP-TF is also highly expressed in RA-sensitive bladder cancer and breast cancer cell lines (data not shown), suggesting that the effect of COUP-TF is not restricted to lung cancer cells. Our studies also reveal that expression of nur77 is associated with retinoid resistance in lung cancer cells (Figure 8). Since nur77 expression is induced rapidly by growth factors and a cAMP-dependent pathway (Hazel et al., 1988; Milbrandt, 1988; Lim et al., 1995), uncontrolled growth signaling in cancer cells may

lead to overexpression of nur77, that in turn may cause retinoid resistance through inhibition of COUP-TF activity. Hence, the studies described here provide an important mechanism by which retinoid sensitivity is regulated in cancer cells.

We have shown recently that up-regulation of RAR $\beta$  expression by RA correlates with RA-induced growth inhibition in human breast cancer and lung cancer cell lines (Zhang *et al.*, 1996; Liu *et al.*, 1996). In RA-sensitive cancer cell lines, expression of RAR $\beta$  is strongly induced by RA. In contrast, RA had little effect on RAR $\beta$  expression in RA-resistant cancer cell lines (Zhang *et al.*, 1996; Liu *et al.*, 1996). *In vivo*, the clinical response of patients with oral dysplasia to RA is associated with inducibility of RAR $\beta$  (Lotan *et al.*, 1995). The  $\beta$ RARE present in the RAR $\beta$  promoter mediates the induction of RAR $\beta$  by RA (de The *et al.*, 1990; Hoffmann *et al.*, 1990; Sucov *et al.*, 1990), and is activated mainly by RAR/RXR heterodimers (Zhang *et al.*, 1992a; Valcarcel *et al.*, 1994). We have observed previously that RAR $\beta$  cannot be induced by RA in many human lung cancer cell lines even though RAR and RXR are expressed (Zhang *et al.*, 1994). Our present finding that the relative concentrations of COUP-TF and nur77 are involved in the regulation of RAR $\beta$  inducibility by RA through their modulation of  $\beta$ RARE activity provides an explanation of retinoid refractoriness in inducing RAR $\beta$  observed in the lung cancer cell lines. COUP-TF is only expressed in lung cancer cell lines in which the  $\beta$ RARE is highly sensitive to RA (Figure 8), suggesting that it is required for maintaining the sensitivity of the  $\beta$ RARE to RA. The effect of COUP-TF is likely to be mediated by its binding to the  $\beta$ RARE, as demonstrated by our finding that COUP-TF expressed in RA-sensitive Calu-6 lung cancer cells formed a strong  $\beta$ RARE-binding complex that was not observed in RA-resistant H292 cells (Figure 10). The observation that RAR $\beta$  is highly induced by RA in Calu-6 cells (Figure 9A) indicates that the binding of COUP-TF to the  $\beta$ RARE does not interfere with RA-induced retinoid receptor activity. This is also supported by *in vivo* observations (Reuberte *et al.*, 1993; Lutz *et al.*, 1994) that RAR $\beta$  is expressed in motor neurons at the time when COUP-TF is expressed. Hence, the expression levels we observed in various cancer cell lines do not function to inhibit RA-induced RAR $\beta$  expression, but repress RAR $\beta$  expression in the absence of RA. In this study, we also found that nur77 is highly expressed in RA-resistant lung cancer cell lines (Figure 8). Thus, the loss of RA inducibility of RAR $\beta$  expression in certain lung cancer cell lines, such as H292, is not due to abnormal expression and function of RARs and RXRs that activate the  $\beta$ RARE, but to lack of COUP-TF and/or overexpression of nur77 that modulates basal levels of RAR $\beta$  expression. Such a loss of RA sensitivity in lung cancer cells can be restored by expression of COUP-TF, as demonstrated by our transient transfection (Figure 9) and stable transfection (Figure 11) of COUP-TF in RA-resistant H292 cells. Interestingly, an increase in RAR $\beta$  inducibility by stable expression of COUP-TF is also accompanied by an enhancement of growth inhibition by RA (Figure 11). This observation further supports our previous finding that induction of RAR $\beta$  by RA is involved in RA-induced growth inhibition in breast cancer cell lines (Liu *et al.*, 1996).

In summary, the studies described here reveal a novel mechanism that regulates RA sensitivity in cancer cells through heterodimerization of nur77 and COUP-TF. Our data demonstrate that a dynamic equilibrium of the two orphan receptors plays a crucial role in the control of inducibility of RAR $\beta$  expression and growth inhibition by RA in human lung cancer cell lines. Such a mechanism may also be involved in the regulation of the RA sensitivity program during development and in adult life. Since the expression of nur77 is induced by growth signaling (Hazel *et al.*, 1988; Milbrandt, 1988; Lim *et al.*, 1995) while the expression of COUP-TF can be enhanced by RA (Jonk *et al.*, 1994), heterodimerization of nur77 and COUP-TF may mediate 'cross-talk' between growth and vitamin A signalings. Overexpression of nur77 and/or lack of COUP-TF as seen in certain human lung cancer cells may be responsible for RA resistance, and may contribute to cell proliferation and neoplastic transformation by releasing the inhibitory effect of RA on cell growth. Our demonstration that expression of COUP-TF in RA-resistant H292 cells could enhance their RA response provides novel approaches for restoring RA sensitivity in certain RA-resistant cancer cells.

## Materials and methods

### Cell culture

CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Calu-6 and SK-MES-1 cells were maintained in minimum essential medium (MEM) supplemented with 10% FCS. H292, H520, H460, H596, H441 and H661 cells were grown in RPMI 1640 supplemented with 10% FCS. A-549 cells were maintained in F12 medium supplemented with 10% FCS.

### Plasmid constructions

Nur77 expression vectors pECE-nur77, pBluescript-nur77, pGEX-2T-nur77 and pGAD424-nur77 were constructed by cloning the nur77 cDNA (Chang and Kokontis, 1988) fragment into pECE, pBluescript, pGex-2T and yeast vector pGAD424, respectively. The internal *Sst*I fragment was removed from nur77 to generate  $\Delta$ nur77-1. For construction of  $\Delta$ COUP-TFI-1, the internal *Nar*I fragment was removed. The deleted COUP-TF was filled in and religated. To obtain the deletion mutants  $\Delta$ nur77-2 and  $\Delta$ COUP-TFI-2, the *Pst*I site in nur77 and the *Sall* site in COUP-TFI were used to delete the C-terminal fragments. The construction of the reporter plasmids  $\beta$ RARE-tk-CAT, TREpal-tk-CAT, CRBPI-RARE-tk-CAT, CRBPII-RARE-tk-CAT, ApoAI-RARE-tk-CAT, lactoferrin-RARE-tk-CAT and MHC-TRE-HC-CAT has been described previously (Tran *et al.*, 1992; Zhang *et al.*, 1992a,b; Lee *et al.*, 1995). The reporter  $\Delta\beta$ RARE-tk-CAT was obtained by inserting one copy of mutated  $\beta$ RARE oligonucleotide (TGTAGGGTTCACTGAGTTCACTCA) (underlining indicates the mutated nucleotides) into the *Bam*H site of pBLCAT<sub>2</sub> (Luckow and Schutz, 1987). The RAR $\beta$  promoter (*Sma*I-*Eco*RI fragment) reporter has been described (Hoffmann *et al.*, 1990). The construction of COUP-TFI cDNA in the pRc/CMV vector (Invitrogen, San Diego, CA) followed the procedure described previously (Liu *et al.*, 1996).

### Preparation of receptor proteins

Receptor proteins were synthesized by an *in vitro* transcription-translation system using rabbit reticulocyte lysate (Promega) as described previously (Zhang *et al.*, 1992a). The relative amount of the translated proteins was determined using [<sup>35</sup>S]methionine-labeled protein on SDS-PAGE, quantitating the amount of incorporated radioactivity, and normalizing it relative to the content of methionine in each protein.

### Transient and stable transfection assay

CV-1 cells were plated at  $1 \times 10^5$  cells per well in a 24-well plate 16–24 h before transfection as described previously (Zhang *et al.*, 1992a). For Calu-6 and H292 cells,  $5 \times 10^5$  cells were seeded in six-well culture plates. A modified calcium phosphate precipitation procedure was used for transient transfection and is described elsewhere (Zhang *et al.*,

1992a). Briefly, 100 ng of reporter plasmid, 150 ng of  $\beta$ -galactosidase expression vector (pCH 110, Pharmacia) and various amounts of nur77 expression vector were mixed with carrier DNA (pBluescript) to 1000 ng of total DNA per well. CAT activity was normalized for transfection efficiency to the corresponding  $\beta$ -gal activity. For stable transfection, the pRc/CMV-COUP-TFI recombinant plasmid was stably transfected into H292 cells using the calcium phosphate precipitation method, and screened using G418 (GIBCO BRL, Grand Island, NY) as described (Liu et al., 1996).

#### Gel retardation assay

The gel retardation assay using *in vitro* synthesized proteins has been described previously (Zhang et al., 1992a). When interaction of nur77 and COUP-TF was studied, they were incubated on ice for 10 min before performing gel retardation in order to prevent the formation of the COUP-TF homodimer. In most cases, co-translation of nur77 and COUP-TF resulted in much more efficient dimerization of the two proteins. When antibodies were used in the gel retardation assay, 1  $\mu$ l of anti-nur77 (Santa Cruz Biotech., Inc., Santa Cruz, CA) or 1  $\mu$ l of anti-RXR (Lee et al., 1995) was incubated with receptor protein at room temperature for 30 min prior to performing the gel retardation assay. The oligonucleotides used for the gel retardation assay have been described elsewhere (Tran et al., 1992; Zhang et al., 1992a,b; Lee et al., 1995).

#### Immunoprecipitation assay

For the immunoprecipitation assay (Zhang et al., 1992a), 5  $\mu$ l of reticulocyte lysate containing *in vitro* translated  $^{35}$ S-labeled COUP-TFI or COUP-TFII were incubated with 20  $\mu$ l of *in vitro* translated nur77 in 100  $\mu$ l of buffer containing 50 mM KCl and 10% glycerol for 15 min on ice. The reactions were then incubated with 5 ml of anti-nur77 antibody or non-specific pre-immune serum for 2 h on ice. When the peptide from which anti-nur77 antibody (Santa Cruz Biotech., Inc., Santa Cruz, CA) was generated was used, anti-nur77 antibody was incubated with 5  $\mu$ l of peptide at room temperature for 30 min before adding to the reaction mixtures. Immunocomplexes were precipitated by adding 40  $\mu$ l of protein A-Sepharose slurry and mixing continuously in the cold room for 1 h. The complexes were then washed five times with RIPA buffer, resuspended in SDS sample buffer containing 15%  $\beta$ -mercaptoethanol, boiled and resolved by SDS-PAGE.

#### GST pull down assay

To prepare GST-nur77 fusion protein, the nur77 cDNA was cloned in-frame into the expression vector pGEX-2T (Pharmacia). The fusion protein was expressed in bacteria using the procedure provided by the manufacturer, and was analyzed by gel retardation assay and Western blot (data not shown). To analyze the interaction between nur77 and COUP-TF, the fusion protein was immobilized on glutathione-Sepharose beads. For control, the vector protein (GST) prepared under the same conditions was also immobilized. The beads were pre-incubated with bovine serum albumin (1 mg/ml) at room temperature for 5 min.  $^{35}$ S-Labeled *in vitro* synthesized receptor proteins (2–5  $\mu$ l, depending on translation efficiency) were then added to the beads. The beads were then rocked continuously for 1 h at 4°C in a final volume of 200  $\mu$ l in EBC buffer (140 mM NaCl, 0.5% NP-40, 100 mM NaF, 200  $\mu$ M sodium orthovanadate and 50 mM Tris, pH 8.0). After washing five times with NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, 0.5% NP-40), the bound proteins were analyzed by SDS-PAGE.

#### Two-hybrid assay

For the yeast two-hybrid assay, the yeast two-hybrid system from Clontech Inc. (Palo Alto, CA) was used. Nur77 cDNA and deletion mutants were cloned into the yeast expression vector pGAD424 to generate an in-frame fusion with the Gal4 activation domain. COUP-TF cDNA and deletion mutants were cloned into pGBT-9 to produce an in-frame fusion with the Gal4 DBD. The yeast reporter strain Y190 containing a LacZ reporter plasmid with the Gal4 binding site was used for transformation.  $\beta$ -Galactosidase activity was determined following the conditions provided by the manufacturer to assess the interaction between nur77 and COUP-TF.

#### MTT assay

To determine the effect of all-trans RA on the viability of the stable transfectants, cells were seeded at 1000 cells per well in a 96-well plate, and treated with various concentrations of all-trans RA for 6 days. Media were changed every 48 h. The number of viable cells was determined by MTT assay as described previously (Liu et al., 1996).

#### Northern blot

For Northern blot analysis, total RNAs were prepared by the guanidine hydrochloride ultracentrifugation method as described (Zhang et al., 1994). Thirty mg of total RNAs from different cell lines treated with or without 10<sup>-6</sup> M all-trans RA were analyzed by Northern blot. RAR $\beta$ , COUP-TFI or nur77 cDNA were used as probes. To determine that equal amounts of RNA were used, the expression of  $\beta$ -actin was studied.

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#### References

- Baes,M., Gulick,T., Choi,H.-S., Martinoli,M.G., Simha,D. and Moore, D.D. (1994) A new orphan member of the nuclear hormone receptor superfamily that interacts with a subset of retinoic acid response elements. *Mol. Cell. Biol.*, **14**, 1544–1552.
- Baes,M., Castelein,H., Desmet,L. and Declercq,P.E. (1995) Antagonism of COUP-TF and PPAR $\alpha$ /RXR $\alpha$  on the activation of the malic enzyme gene promoter: modulation by 9-cis RA. *Biochem. Biophys. Res. Commun.*, **215**, 338–345.
- Bartel,P.L., Chien,C.-T., Sternglanz,R. and Fields,S. (1993) Using the two-hybrid system to detect protein–protein interactions. In Hartley,D.A. (ed.), *Cellular Interactions in Development: A Practical Approach*. Oxford University Press, Oxford, pp. 153–179.
- Chang,C. and Kokontis,J. (1988) Identification of a new member of the steroid receptor super-family by cloning and sequence analysis. *Biochem. Biophys. Res. Commun.*, **155**, 971–977.
- Chen,J.-Y., Clifford,J., Zusi,C., Starrett,J., Tortolani,D., Ostrowski,J., Reczek,P.R., Champon,P. and Gronemeyer,H. (1996) Two distinct events in retinoid receptor heterodimer action revealed by ligand synergism. *Nature*, **382**, 819–822.
- Cooney,A.J., Tsai,S.Y., O'Malley,B.W. and Tsai,M.-J. (1992) Chicken ovalbumin upstream promoter transcription factor (COUP-TF) dimers bind to different GGTCA response elements, allowing COUP-TF to repress hormonal induction of the vitamin D<sub>3</sub> thyroid hormone, and retinoic acid receptors. *Mol. Cell. Biol.*, **12**, 4153–4163.
- de The,H., Vivanco-Ruiz,M.M., Tiollais,P., Stunnenberg,H. and Dejean,A. (1990) Identification of a retinoic acid responsive element in the retinoic acid receptor  $\beta$  gene. *Nature*, **343**, 177–180.
- Dey,A., Minucci,S. and Ozato,K. (1994) Ligand-dependent occupancy of the retinoic acid receptor  $\beta$ 2 promoter *in vivo*. *Mol. Cell. Biol.*, **14**, 8191–8201.
- Forman,B.M., Umesono,K., Chen,J. and Evans,R.M. (1995) Unique response pathways are established by allosteric interactions among nuclear hormone receptors. *Cell*, **81**, 541–550.
- Gudas,L.J., Sporn,M.B. and Roberts,A.B. (1994) Cellular biology and biochemistry of the retinoids. In Sporn,M.B., Roberts,A.B. and Goodman,D.S. (eds), *The Retinoids*. 2nd edn. Raven Press, New York, pp. 443–520.
- Hazel,T.G., Nathans,D. and Lau,L.F. (1988) A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. *Proc. Natl Acad. Sci. USA*, **85**, 8444–8448.
- Hoffmann,B., Lehmann,J.M., Zhang,X.-k., Hermann,T., Graupner,G. and Pfahl,M. (1990) A retinoic acid receptor-specific element controls the retinoic acid receptor- $\beta$  promoter. *Mol. Endocrinol.*, **4**, 1734–1743.
- Hong,W.K. and Itri,L.M. (1994) Retinoids and human cancer. In Sporn,M.B., Roberts,A.B. and Goodman,D.S. (eds), *The Retinoids*. 2nd edn. Raven Press, New York, pp. 597–630.
- Jonk,L.J.C., de Jonge,M.E., Pals,C.E.G.M., Wissink,S., Vervaart,J.M., Schoolemmert,J. and Kruijer,W. (1994) Cloning and expression during development of three murine members of the COUP family of nuclear orphan receptors. *Mech. Dev.*, **47**, 81–87.
- Kastner,P., Mark,M. and Champon,P. (1995) Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell*, **83**, 859–869.
- Kim,Y.-H. et al. (1995) Retinoid refractoriness occurs during lung carcinogenesis despite functional retinoid receptors. *Cancer Res.*, **55**, 5603–5610.

- Kliewer,S.A., Kazuhiko,U., Heyman,R.A., Mangelsdorf,D.J., Dyck,J.A. and Evans,R.M. (1992) Retinoid X receptor-COUP-TF interactions modulate retinoic acid signaling. *Proc. Natl Acad. Sci. USA*, **89**, 1448–1452.
- Ladias,J.A. and Karathanasis,S.K. (1991) Regulation of the apolipoprotein AI gene by ARP-1, a novel member of the steroid receptor superfamily. *Science*, **251**, 561–565.
- Leblanc,B.P. and Stunnenberg,H.G. (1995) 9-cis retinoic acid signaling changing partners causes some excitement. *Genes Dev.*, **9**, 1811–1816.
- Lee,M.-O., Liu,Y. and Zhang,X.-k. (1995) A retinoic acid response element that overlaps an estrogen response element mediates multihormonal sensitivity in transcriptional activation of the lactoferrin gene. *Mol. Cell. Biol.*, **15**, 4194–4207.
- Lim,R.W., Zhu,C.Y. and Stringer,B. (1995) Differential regulation of primary response gene expression in skeletal muscle cell through multiple signal transduction pathways. *Biochim. Biophys. Acta*, **1266**, 91–100.
- Liu,Y., Lee,M.-O., Wang,H.-G., Li,Y., Hashimoto,Y., Klaus,M., Reed,J.C. and Zhang,X.-k. (1996) RAR $\beta$  mediates the growth inhibitory effect of retinoic acid by inducing cell apoptosis in human breast cancer cells. *Mol. Cell. Biol.*, **16**, 1138–1149.
- Lotan,R., Xu,X.-C., Lippman,S.M., Ro,J.Y., Lee,J.S., Lee,J.J. and Hong,W.K. (1995) Suppression of retinoic acid receptor- $\beta$  in premalignant oral lesions and its up-regulation by isotretinoin. *N. Engl. J. Med.*, **332**, 1405–1410.
- Luckow,B. and Schutz,G. (1987) CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. *Nucleic Acids Res.*, **15**, 5490.
- Lutz,B., Kuratani,S., Cooney,A.J., Wawersik,S., Tsai,S.Y., Eichele,G. and Tsai,M.-J. (1994) Developmental regulation of the orphan receptor COUP-TFII gene in spinal motor neurons. *Development*, **120**, 25–36.
- Mangelsdorf,D.J. and Evans,R.M. (1995) The RXR heterodimers and orphan receptors. *Cell*, **83**, 841–850.
- Milbrandt,J. (1988) Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. *Neuron*, **1**, 183–188.
- Miyajima,N., Kadouki,Y., Fukushige,S., Shimizu,S., Semba,K., Yamanashi,Y., Matsubara,K., Toyoshima,K. and Yamamoto,T. (1988) Identification of two novel members of the erb A superfamily by molecular cloning: the gene products of the two are highly related to each other. *Nucleic Acids Res.*, **16**, 11057–11074.
- Perlmann,T. and Jansson,L. (1995) A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and Nurr1. *Genes Dev.*, **9**, 769–782.
- Reuberte,E., Friederich,V., Champon,P. and Morrissey,G. (1993) Retinoic acid receptors and cellular retinoid binding proteins. III. Their differential transcript distribution during mouse nervous system development. *Development*, **118**, 267–282.
- Sucov,H.M., Murakami,K.K. and Evans,R.M. (1990) Characterization of an autoregulated response element in the mouse retinoic acid receptor type  $\beta$  gene. *Proc. Natl Acad. Sci. USA*, **87**, 5392–5396.
- Tran,P., Zhang,X.-k., Salbert,G., Hermann,T., Lehmann,J.M. and Pfahl,M. (1992) COUP orphan receptors are negative regulators of retinoic acid response pathways. *Mol. Cell. Biol.*, **12**, 4666–4676.
- Valcarcel,R., Holz,H., Jimenez,C.G., Baretto,D. and Stunnenberg,H.G. (1994) Retinoid-dependent *in vitro* transcription mediated by the RXR/RAR heterodimer. *Genes Dev.*, **8**, 3068–3079.
- van der Leede,B.M., van den Brink,C.E. and van der Saag,P.T. (1993) Retinoic acid receptor and retinoid X receptor expression in retinoic acid-resistant human tumor cell lines. *Mol. Carcinogen.*, **8**, 112–122.
- Wang,L.-H., Tsai,S.Y., Cook,R.G., Beattie,W.G., Tsai,M.-J. and O’Malley,B.W. (1989) COUP transcription factor is a member of the steroid receptor superfamily. *Nature*, **340**, 163–166.
- Warrell,R.P.Jr., de The,H., Wang,Z.Y. and Degos,L. (1993) Acute promyelocytic leukemia. *N. Engl. J. Med.*, **329**, 177–189.
- Widom,R.L., Rhee,M. and Karathanasis,S.K. (1992) Repression by ARP-1 sensitizes apolipoprotein AI gene responsiveness to RXR $\alpha$  and retinoic acid. *Mol. Cell. Biol.*, **12**, 3380–3389.
- Wilson,T.E., Fahrner,T.J., Johnston,M. and Milbrandt,J. (1991) Identification of the DNA binding site for NGFI-B by genetic selection in yeast. *Science*, **252**, 1296–1300.
- Williams,G.T. and Lau,L.F. (1993) Activation of the inducible orphan receptor gene nur77 by serum growth factors: dissociation of immediate-early and delayed-early responses. *Mol. Cell. Biol.*, **13**, 6124–6136.
- Zhang,X.-k. and Pfahl,M. (1993) Regulation of retinoid and thyroid hormone action through homodimeric and heterodimeric receptors. *Trends Endocrinol. Metab.*, **4**, 156–162.
- Zhang,X.-k., Hoffmann,B., Tran,P., Graupner,G. and Pfahl,M. (1992a) Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. *Nature*, **355**, 441–446.
- Zhang,X.-k., Lehmann,J., Hoffmann,B., Dawson,M., Cameron,J., Graupner,G., Hermann,T., Tran,P. and Pfahl,M. (1992b) Homodimer formation of retinoid X receptor induced by 9-cis retinoic acid. *Nature*, **358**, 587–591.
- Zhang,X.-k., Liu,Y., Lee,M.-O. and Pfahl,M. (1994) A specific defect in the retinoic acid receptor associated with human lung cancer cell lines. *Cancer Res.*, **54**, 5663–5669.
- Zhang,X.-k., Liu,Y. and Lee,M.-O. (1996) Retinoid receptors in human lung cancer and breast cancer. *Mutat. Res.*, **350**, 267–277.

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# Retinyl Methyl Ether Down-Regulates Activator Protein 1 Transcriptional Activation in Breast Cancer Cells<sup>1</sup>

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## ABSTRACT

Retinyl methyl ether (RME) is known to prevent the development of mammary cancer. However, the mechanism by which RME exerts its anticancer effect is presently unclear. The diverse biological functions of retinoids, the vitamin A derivatives, are mainly mediated by their nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). RARs and RXRs are ligand-dependent transcriptional factors that either activate gene transcription through their binding to retinoic acid response elements or repress transactivation of genes containing the activator protein 1 (AP-1) binding site. Previous studies demonstrated that RME can modulate transcriptional activity of retinoid receptors on retinoic acid response elements, suggesting that regulation of retinoid receptor activity may mediate the anticancer effect of RME. In this study, we present evidence that RME can down-regulate AP-1 activity induced by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate, insulin, growth factors, and the nuclear proto-oncogenes c-Jun and c-Fos. Transient transfection assays demonstrate that inhibition of AP-1 activity occurs on the human collagenase promoter containing an AP-1 binding site or the thymidine kinase promoter linked with an AP-1 binding site. In HeLa cells, the inhibition is observed when RAR- $\alpha$  and/or RXR- $\alpha$  but not RAR- $\beta$  or RAR- $\gamma$  expression vectors are cotransfected, whereas the endogenous retinoid receptors in breast cancer cells T-47D and ZR-75-1 were sufficient to confer the inhibition by RME. Furthermore, using gel retardation assay, we show that 12-O-tetradecanoylphorbol-13-acetate- and epidermal growth factor-induced AP-1 binding activity in breast cancer cells is inhibited by RME. These results suggest that one of the mechanisms by which RME prevents cancer development may be due to the repression of AP-1-responsive genes.

## INTRODUCTION

Retinoids are a group of natural and synthetic vitamin A analogues that exert profound effects on many biological processes, including cell proliferation and differentiation, vision, reproduction, morphogenesis, and pattern formation (1–2). They are promising agents for the prevention of human cancers (3, 4), including cancers of the breast, skin, head and neck, lung, prostate, and bladder. Retinyl ethers represent an unique class of vitamin A analogues with effective cancer-preventive function (5–7). RME<sup>3</sup> is significantly more active than RA in inhibiting hyperplasia induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in mouse prostate organ culture (8) and in inhibiting hyperplasia and squamous metaplasia induced by different carcinogens in organ cultures of hamster trachea (9). RME and related compounds prevent the development of mammary tumors induced in

rats by different carcinogens (5–7). Studies of their pharmacological distribution in tissue reveal a correlation between their accumulation and their cancer-preventive effect (6, 10). However, the molecular mechanism by which RME exerts its anticancer effect remains unknown.

The effects of retinoids are thought to result from modulation of gene activity by at least two distinct classes of nuclear receptors: (a) RARs, which bind and are activated by *trans*-RA or 9-*cis*-RA; and (b) RXRs, which bind only to the 9-*cis*-RA isomer (11–13). These receptors exist as major subtypes  $\alpha$ ,  $\beta$ , and  $\gamma$ , from which multiple isoforms can be generated as a result of alternative splicing and differential promoter usage (11–13). RARs and RXRs, like other members of the steroid/thyroid hormone superfamily, have similar domain structures: an autonomous activation domain (AF-1) in the amino-terminal part of the receptor (A/B region), and a hormone-binding domain (E region) responsible for ligand binding and receptor transactivation (11, 13). RARs and RXRs function as ligand-activated transcription factors that bind to specific RAREs on target genes as homodimeric or heterodimeric complexes and positively or negatively regulate the expression of the genes (11–14). Retinoid receptor-mediated transcriptional activation can contribute to the anticancer activity of retinoids. A well-known example is the effect of RA-activated RAR- $\alpha$  in the treatment of acute promyelocytic leukemia, whereby the proliferating nondifferentiated leukemic promyelocyte matures into a nonproliferating differentiated granulocyte on retinoid treatment (15). Furthermore, ligand-activated RAR- $\beta$  may be required for anticancer activity of vitamin A analogues in breast cancer cells (16) or in patients with oral lesions (17).

In addition to the regulation of RARE-containing genes, retinoid receptors in response to RA can inhibit the effect of the tumor promoter TPA by repressing the transcriptional activity of AP-1 (14, 18–20). AP-1 constituents are products of the cellular proto-oncogenes c-Jun and c-Fos that bind as either c-Jun homodimers or c-Jun/c-Fos heterodimers to TREs, resulting in a stimulation of TRE-dependent transcription (21). Unlike the effect of retinoid receptors on RAREs, inhibition of AP-1 activity by retinoid receptors does not involve a direct retinoid receptor-RARE interaction, but rather a protein-protein interaction that may result in the inhibition of AP-1 DNA binding. Recent results suggest that the molecular basis of receptor-mediated inhibition of AP-1 transcriptional activation may be due to competition for a common coactivator such as CREB (22). CBP is required for transcriptional activation by both CREB and AP-1 (23–25) as well as by nuclear receptors such as RARs (22). Such interaction between membrane and nuclear receptor signaling pathways mediated by RAR/AP-1 interaction through CBP may be important in understanding the mechanisms underlying the potent antineoplastic effects of retinoids. Because many of the AP-1-responsive genes are involved in cancer cell proliferation and transformation (21), the anti-AP-1 activity of retinoids could contribute to their chemopreventive effect by inhibiting the processes of cell proliferation and cell transformation. This is supported by a recent observation that retinoids that selectively inhibit AP-1 activity and cannot induce transactivation of RARE-containing genes were able to inhibit TPA-induced transformation and the clonal growth of the promotion-

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<sup>3</sup> The abbreviations used are: RME, retinyl methyl ether; AP-1, activator protein 1; CREB, cAMP response element binding protein; CBP, CREB-binding protein; EGF, epidermal growth factor; RA, retinoic acid; RAR, RA receptor; RARE, RA response element; RXR, retinoid X receptor; tk, thymidine kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; TRE, TPA response element;  $\beta$ -gal,  $\beta$ -galactosidase.

sensitive mouse epidermal JB6 cell line (26). In addition, anti-AP-1 retinoids inhibit the proliferation of lung and breast cancer cells (27, 28).

We previously showed that RME could influence the transcriptional potential of RARs and RXRs (29), suggesting that RME may exert its anticancer activity through regulation of retinoid receptor activity. To further extend our understanding of the anticancer effect of RME, we investigated the possibility that RME regulates the anti-AP-1 function of retinoid receptors. Our data demonstrate that RME is an effective inhibitor of AP-1 activity induced by tumor promoter TPA, growth factors, and the nuclear proto-oncogenes c-Jun and c-Fos and suggest that one of the mechanisms by which RME exerts its cancer-preventive action may be due to the repression of AP-1-responsive genes.

## MATERIALS AND METHODS

**Cell Culture and Reagents.** HeLa cells were grown in DMEM supplemented with 10% FCS. Breast cancer cell lines T-47D and ZR-75-1 were maintained in RPMI 1640 supplemented with 10% FCS. Trans-RA, TPA, insulin, and EGF were purchased from Sigma Chemical Co. (St. Louis, MO). Synthesis of RME was accomplished as described previously (7). 9-cis-RA was kindly provided by Dr. M. I. Dawson (SRI International, Menlo Park, CA).

**Plasmids.** The collagenase promoter reporter construct ( $-73\text{Col-CAT}$ ) was described previously (18, 30). The  $\text{TRE}_2\text{-tk-CAT}$  reporter was constructed by inserting two copies of AP-1 binding site (TRE; 5'-AGCTTGACTCATCCGGATCCGGATGAGTGAGTCACCAAGCT-3') present in the collagenase promoter region into the *Bam*H site of pBL-CAT<sub>2</sub> (31). Expression vectors for RAR- $\alpha$ , RAR- $\beta$ , RAR- $\gamma$ , RXR- $\alpha$ , c-Jun, and c-Fos were described previously (18, 32).

**Transient Transfection and CAT Assay.** HeLa cells were plated at a density of  $10^5$  cells/well in 24-well plates and transfected by a modified calcium phosphate precipitation procedure (18). The precipitates contained 100 ng of reporter plasmid, 100 ng of  $\beta$ -gal expression vector (pCH 110; Pharmacia), and variable amounts of receptor expression vectors. The total amount of transfected DNA was adjusted with pBluescript (Stratagene) to 1  $\mu$ g. For cancer cell lines, cells were seeded at a density of  $5 \times 10^5$  cells/well in 6-well plates. The precipitates contained 250 ng of reporter plasmid and 400 ng of  $\beta$ -gal expression vector. The total amount of transfected DNA was adjusted with pBluescript to 2.5  $\mu$ g. Twenty-four h after transfection, cells were incubated in a medium containing 0.5% charcoal-treated FCS with retinoids and/or TPA (100 ng/ml), EGF (10 ng/ml), or insulin (10  $\mu$ g/ml) for an additional 16 h. CAT activity was determined by using [<sup>3</sup>H]acetyl-CoA as substrate.  $\beta$ -gal activities were measured to normalize transfection efficiency.

**Preparation of Nuclear Extracts and Gel Retardation Assays.** Nuclear extracts were prepared essentially according to the method described previously (33). Briefly, cells growing in 0.5% charcoal-treated FCS were treated with retinoids ( $10^{-6}$  M *trans*-RA or RME) and/or TPA (100 ng/ml) or EGF (10 ng/ml) for 6 h. Cells were washed with ice-cold PBS solution and scraped into PBS using a rubber policeman. The cells were pelleted by low-speed centrifugation and resuspended in 1 ml of buffer A [10 mM Tris-HCl (pH 7.4), 3 mM CaCl<sub>2</sub>, and 2 mM MgCl<sub>2</sub>]. After pelleting, the cells were lysed in 1 ml of buffer A containing 1% NP40 by 10–15 strokes using an ice-cold Dounce homogenizer. Nuclei were collected by centrifugation at 2000  $\times g$  and washed with 1 ml of buffer B [10 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, and 0.5 mM DTT]. Nuclear proteins were extracted with 200  $\mu$ l of high-salt buffer C [20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, and 0.5 mM DTT]. All buffers contained protease inhibitors, *i.e.*, 100  $\mu$ g/ml phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml leupeptin, and 1  $\mu$ g/ml aprotinin. To study AP-1 binding, 3  $\mu$ g of nuclear extracts were analyzed by gel retardation assay using the <sup>32</sup>P-labeled AP-1 binding site as a probe as described previously (18). The AP-1 used in the experiments is the TRE present in collagenase promoter (TGACTCA). For control, binding of nuclear proteins to SP-1 (CCCGAGGTGGCGGGTGAGGCC) was used. Labeled DNA probes were purified by gel electrophoresis and used for the gel retardation assay.

## RESULTS

**Down-Regulation of TPA-induced Collagenase Promoter Activity by RME.** Retinoid receptors, in response to their ligands, can down-regulate the activities of the tumor promoter TPA and oncogenes c-Jun and c-Fos (18–20). We have recently observed that RME can modulate transcriptional activity of retinoid receptors on RAREs (29). To determine whether RME could also influence the anti-AP-1 activity of retinoid receptors, the  $-73\text{Col-CAT}$  reporter gene (18, 30) was transfected with or without RARs ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) and/or RXR- $\alpha$  expression vectors into HeLa cells, which express very low levels of retinoid receptors (18). In these cells, the transactivation activity of the collagenase promoter can be induced by TPA due to the presence of an AP-1 binding site (TRE; Ref. 30). After transfection, cells were treated with 100 ng/ml TPA in the absence or presence of various concentrations ( $10^{-8}$ ,  $10^{-7}$ , and  $10^{-6}$  M) of RME. For comparison,

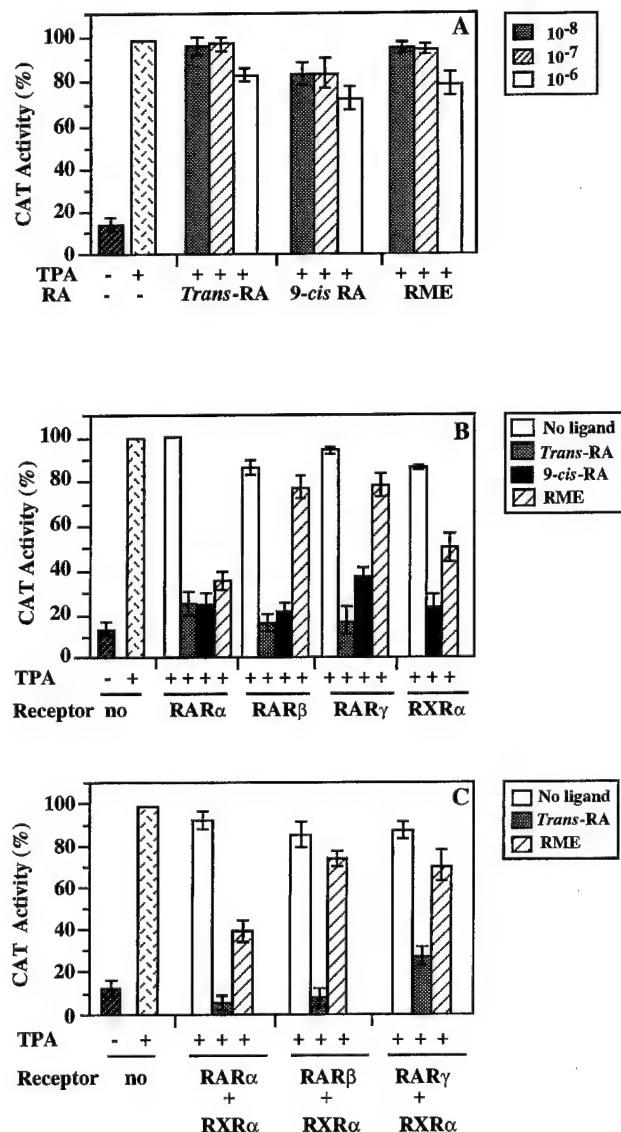


Fig. 1. Inhibition of TPA-induced collagenase promoter activity by RME. The  $-73\text{Col-CAT}$  reporter (100 ng) was cotransfected without (A) or with the indicated retinoid receptor expression vectors (200 ng; B and C) into HeLa cells. After transfection, cells were incubated in DMEM containing 0.5% FCS and treated with either *trans*-RA, 9-cis-RA, or RME and/or TPA (100 ng/ml). Twenty-four h later, the cells were harvested, and CAT activities were determined.  $\beta$ -gal activities were measured to normalize transfection efficiency. The mean of CAT activity in at least three independent experiments is shown ( $\pm$  SE).

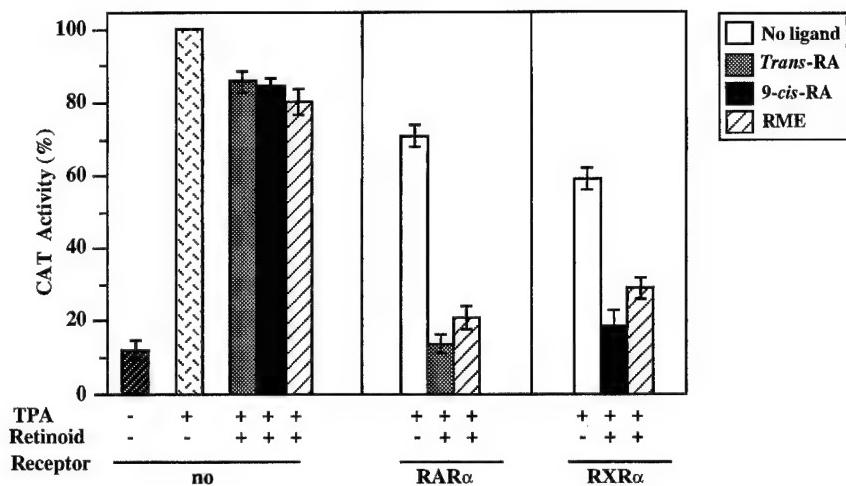


Fig. 2. Inhibition of TPA-induced TRE-tk-CAT activity by RME. The TRE<sub>2</sub>-tk-CAT reporter (100 ng) was cotransfected with or without RAR- $\alpha$  or RXR- $\alpha$  expression vectors (200 ng) into HeLa cells. After transfection, the cells were incubated in DMEM containing 0.5% FCS and treated with either 10<sup>-6</sup> M *trans*-RA, 9-cis-RA, or RME and/or TPA (100 ng/ml). CAT activities were measured as described in the legend to Fig. 1. Data shown represent mean of duplicate experiments ( $\pm$  SE).

the effect of *trans*-RA and 9-cis-RA was examined. Treatment of HeLa cells with TPA led to a 7-fold induction of promoter activity (Fig. 1), presumably through induction of endogenous AP-1 activity (30). In the absence of cotransfected RA receptors, *trans*-RA, 9-cis-RA, or RME at 10<sup>-8</sup> and 10<sup>-7</sup> M did not show a clear effect on TPA-induced collagenase promoter activity (Fig. 1A). At 10<sup>-6</sup> M, all three retinoids showed slight inhibition of TPA-induced reporter activity with 20, 30 and 25% inhibition, respectively (Fig. 1A). When RAR- $\alpha$  expression vector was cotransfected, both *trans*-RA and 9-cis-RA at 10<sup>-6</sup> M completely inhibited TPA-induced collagenase promoter activity (Fig. 1B), as reported previously (18). Remarkably, RME at 10<sup>-6</sup> M exhibited a 65% inhibitory effect (Fig. 1B). The inhibition by RME seems to be specific for RAR- $\alpha$ , because cotransfection of RAR- $\beta$  or RAR- $\gamma$  did not lead to inhibition (Fig. 1B). In comparison, *trans*-RA or 9-cis-RA exhibited strong inhibition under the same conditions, with 90 and 85% for RAR- $\beta$  and 90 and 60% for RAR- $\gamma$ , respectively. When RXR- $\alpha$  expression vector was cotransfected (Fig. 1B), both 9-cis-RA and RME showed inhibition (75 and 50%, respectively). RAR and RXR heterodimerization is required for efficient RARE binding and transactivation (11–13, 32). To investigate the possible effect of heterodimerization, each RAR expression vector was cotransfected with RXR- $\alpha$  (Fig. 1C). Our results demonstrate that cotransfection of RXR- $\alpha$  does not significantly influence

the inhibition profile of RME (Fig. 1C). Together, these data demonstrate that RME in the presence of RAR- $\alpha$  and/or RXR- $\alpha$  is an effective inhibitor of TPA-induced collagenase promoter activity.

To determine whether inhibition of TPA-induced activity by RME is mediated by the TRE site and not by other regions of the collagenase promoter, the TRE was cloned into pBL-CAT<sub>2</sub>, which contains the tk promoter linked with the CAT gene (31). The resulting TRE<sub>2</sub>-tk-CAT reporter was transiently transfected into HeLa cells. Treatment of HeLa cells with TPA (Fig. 2) significantly induced the reporter activity (an 8-fold induction) as that observed on -73Col-CAT (Fig. 1), suggesting that the reporter is responsive to the TPA-induced endogenous AP-1 activity. TPA-induced reporter activity was not clearly affected by either 10<sup>-6</sup> M *trans*-RA, 9-cis-RA, or RME when the cells were treated with these compounds. However, all ligands showed a strong inhibitory effect on TPA-induced reporter activity when RAR- $\alpha$  or RXR- $\alpha$  expression vectors were cotransfected (Fig. 2). Under the conditions used, 10<sup>-6</sup> M *trans*-RA completely inhibited TPA-induced reporter activity, whereas about 80% of TPA activity was repressed by 10<sup>-6</sup> M RME in the presence of RAR- $\alpha$  (Fig. 2) and 50% in the presence of RXR- $\alpha$ . Similar to that observed on the collagenase promoter (Fig. 1B), cotransfection of either RAR- $\beta$  or RAR- $\gamma$  did not influence the inhibitory effect of

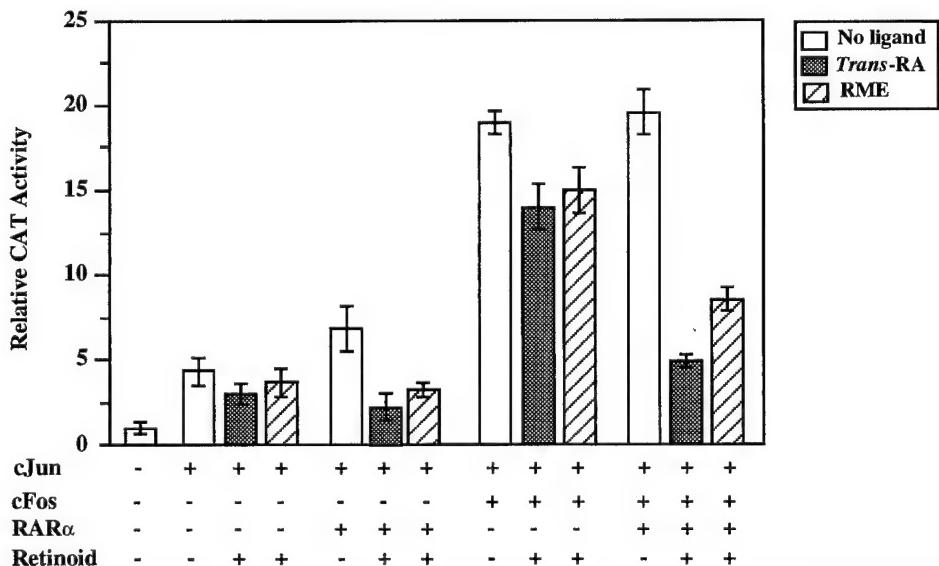


Fig. 3. Inhibition of c-Jun and c-Fos activity by RME. The TRE<sub>2</sub>-tk-CAT reporter (100 ng) was cotransfected with c-Jun alone (50 ng) or together with c-Fos (50 ng) expression vectors in the absence or presence of RAR- $\alpha$  (200 ng) into HeLa cells. After transfection, cells were treated with 10<sup>-6</sup> M *trans*-RA or RME. CAT activities were determined as described in the legend to Fig. 1. Data shown are representative of three independent experiments ( $\pm$  SE).

Fig. 4. Inhibition of TPA-induced collagenase promoter activity by RME in human breast cancer cells. A, T-47D cell line. B, ZR-75-1 cell line. The -73Col-CAT reporter (250 ng) was transfected into T-47D and ZR-75-1 cells. After transfection, cells were incubated in RPMI 1640 containing 0.5% FCS and treated with  $10^{-6}$  M *trans*-RA or RME and/or TPA (100 ng/ml). CAT activities were measured as described in the legend to Fig. 1. Data shown are representative of three independent experiments ( $\pm$  SE).

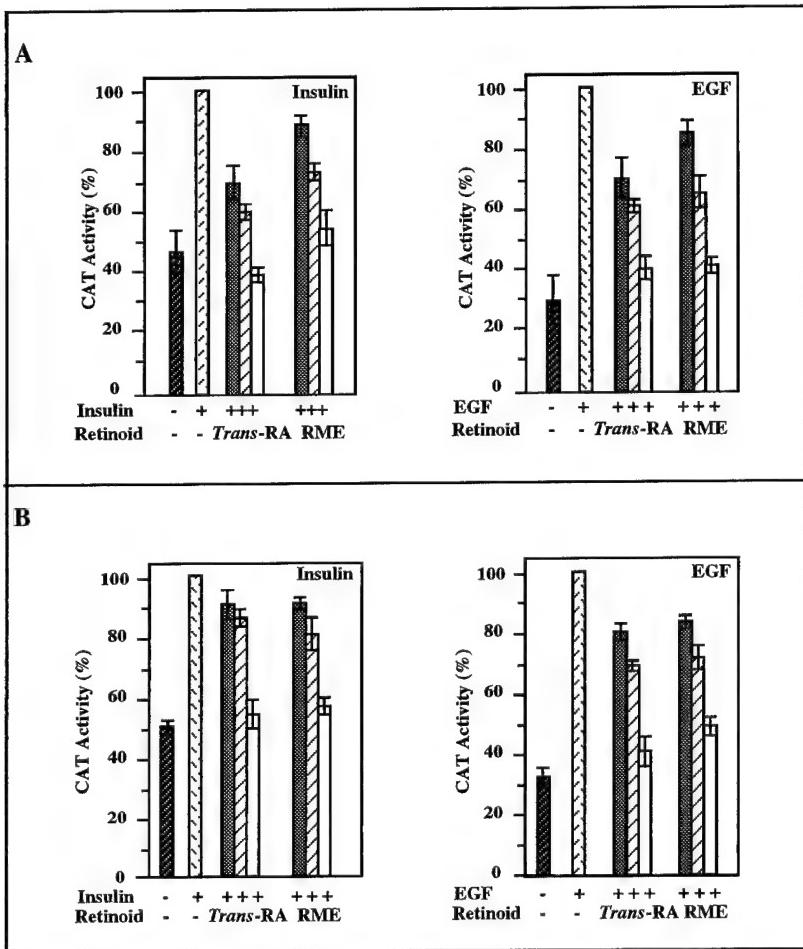
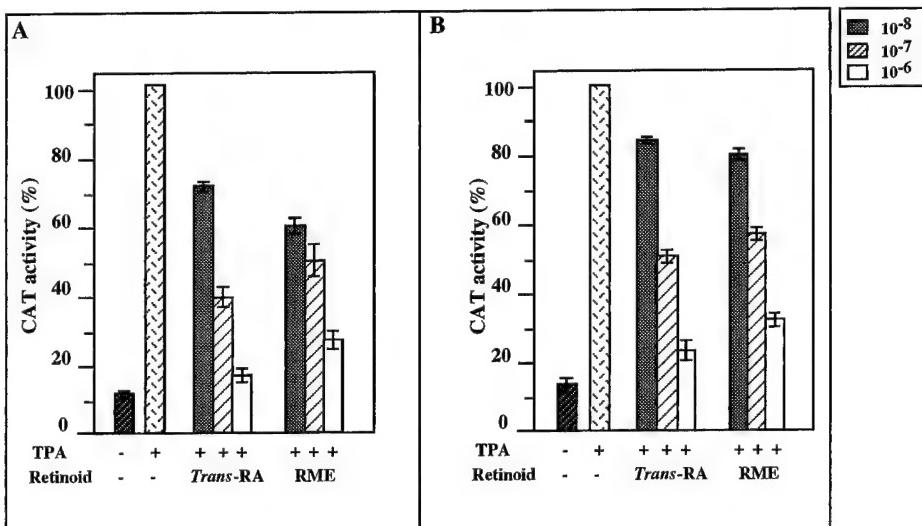


Fig. 5. Inhibition of peptide growth factors-induced collagenase promoter activity in human breast cancer cells. A, T-47D cell line. B, ZR-75-1 cell line. Transfection was carried out as described in the legend to Fig. 4. After transfection, cells were treated with either  $10^{-6}$  M *trans*-RA or  $10^{-6}$  M RME and/or insulin (10  $\mu$ g/ml) or EGF (10 ng/ml). CAT activities were determined as described in the legend to Fig. 1. Data shown represent the mean of duplicate experiments ( $\pm$  SE).

RME (data not shown). Thus, inhibition by RME of TPA activity on the collagenase promoter is mediated by the TRE.

**Effect of RME on the Nuclear Proto-Oncogenes c-Jun and c-Fos.** The observation that TPA-induced TRE activity is inhibited by RME suggests that RME may inhibit the proto-oncogenes c-Jun and c-Fos that bind to the TRE (30). To evaluate this possibility, TRE<sub>2</sub>-tk-CAT was cotransfected with either c-Jun alone or together with c-Fos into HeLa cells. An induction of reporter activity of about 4-fold

was observed when 100 ng of c-Jun expression vector was cotransfected (Fig. 3). When RAR- $\alpha$  expression vector was cotransfected, 50% of the c-Jun homodimer activity was repressed by  $10^{-6}$  M RME. Cotransfection of c-Jun and c-Fos expression vectors largely increased TRE<sub>2</sub>-tk-CAT reporter activity, with a 14-fold induction of reporter gene transcription (Fig. 3). The induced reporter activity was also repressed by RME in the presence of RAR- $\alpha$ . Together, these data demonstrate that RME in the presence of RAR- $\alpha$  can repress

c-Jun homodimer- and c-Jun/c-Fos heterodimer-induced TRE activity. Thus, RME inhibits not only TPA-induced AP-1 activity but also the activity of the proto-oncogenes c-Jun and c-Fos.

#### Inhibition of AP-1 Activity by RME in Human Breast Cancer Cells.

To determine whether RME could inhibit AP-1 activity in breast cancer cells, the -73Col-CAT was transiently transfected into T-47D and ZR-75-1 breast cancer cell lines, both of which express significant levels of RAR- $\alpha$  (16). Fig. 4 shows that treatment of the cells with TPA led to an increase of reporter gene transcription of about 7-fold in both T-47D (Fig. 4A) and ZR-75-1 cells (Fig. 4B). TPA-induced activity was inhibited by either *trans*-RA or RME in a concentration-dependent manner (Fig. 4, A and B). RME ( $10^{-7}$  M) inhibited the induced reporter activities in both cell lines by about 40%, whereas at  $10^{-6}$  M, RME almost completely inhibited the TPA-induced reporter activity. Several peptide growth factors, such as EGF and insulin, exhibit strong mitogenic activity. They have been implicated in breast tumor growth and are thought to contribute to tumor malignancy (34, 35). To study whether RME could also inhibit peptide growth factor-induced AP-1 activity in breast cancer cells, the -73Col-CAT reporter was transfected into both T-47D and ZR-75-1 cells. Transfected cells were then treated with insulin (10  $\mu$ g/ml) or EGF (10 ng/ml; Fig. 5). Treatment of T-47D cells (Fig. 5A) and ZR-75-1 cells (Fig. 5B) with either insulin or EGF resulted in about a 2–3-fold increase of the reporter activity. Similar to its effect on TPA-induced reporter activity, RME also strongly inhibited AP-1 activity induced by either insulin or EGF (Fig. 5, A and B). Thus, endogenous RARs in breast cancer cells are sufficient to mediate the inhibitory effect of RME on the AP-1 activity induced by a variety of stimuli.

#### Effect of RME on TPA- and EGF-induced AP-1 DNA Binding Activity.

Inhibition of AP-1 binding by RARs has been suggested to account for the anti-AP-1 effect of retinoids (18–20). To investigate whether inhibition of AP-1 activity by RME was due to a decrease of AP-1 binding, nuclear proteins were prepared from HeLa cells treated with TPA (100 ng/ml) and/or  $10^{-6}$  M RME. AP-1 binding was then determined by gel retardation assay using the radiolabeled TRE as a probe. Treatment of HeLa cells with TPA strongly enhanced AP-1 binding activity, whereas treatment with RME did not affect AP-1 binding (Fig. 6). When the cells were treated with both TPA and RME, we did not observe a clear inhibition of TPA-induced AP-1 binding activity. Because the inhibitory effect of RME requires retinoid receptors, we therefore examined whether RME could alter AP-1 binding properties in ZR-75-1 breast cancer cells, which express high levels of RAR- $\alpha$  (16). Similar to that observed in HeLa cells, treatment of ZR-75-1 cells with either TPA or EGF strongly induced AP-1 binding activity (Fig. 7). However, when ZR-75-1 cells were pretreated with  $10^{-6}$  M RME, both TPA- and EGF-induced AP-1 binding activities were repressed. These data suggest that the inhibition of expression of AP-1 binding proteins or their binding to the AP-1 site is likely to be responsible for the observed inhibition of AP-1 transcriptional activity by RME.

## DISCUSSION

RME is known to prevent cancer development (5–9). However, the mechanism by which RME exerts its anticancer effect is presently unclear. We have previously examined the effect of RME on the transactivation potential of various retinoid receptors on RAREs and found that RME could modulate the transcriptional activity of retinoid receptors on RAREs (29). Although the biological significance of this effect remains to be seen, the observation that RME could regulate RAR activities led us to study whether RME could modulate the anti-AP-1 effect of retinoid receptors. Our data convincingly demon-

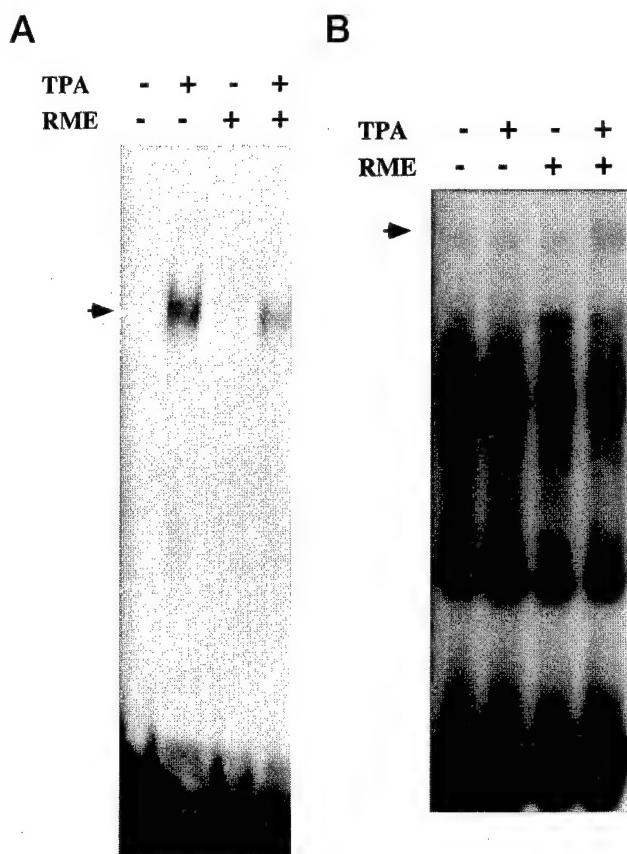


Fig. 6. RME does not inhibit TPA-induced AP-1 binding activity in HeLa cells. Nuclear proteins were prepared from HeLa cells treated with TPA (100 ng/ml) and/or  $10^{-6}$  M RME. A, AP-1 binding of the nuclear proteins was analyzed by gel retardation assay using the  $^{32}$ P-labeled AP-1 binding site (TRE present in the collagenase promoter, TGACTCTA) as a probe. Arrow, AP-1 binding complex. B, binding of nuclear proteins to SP-1 is shown for control (arrow). The result of the gel shift shown is one of two duplicate experiments.

strate that RME can act as an effective anti-AP-1 retinoid. We found that RME could significantly inhibit AP-1 activity induced by TPA and growth factors, and inhibit the transcriptional activity of the nuclear proto-oncogenes c-Jun and c-Fos in HeLa cells (Figs. 1–3) and breast cancer cells (Figs. 4 and 5). Inhibition of AP-1 activity by RME seemed to require RAR- $\alpha$  and/or RXR- $\alpha$  but not RAR- $\beta$  or RAR- $\gamma$ , as demonstrated by cotransfection assays in HeLa cells (Fig. 1). Importantly, endogenous RARs in breast cancer cells were able to mediate the anti-AP-1 activity of RME (Figs. 4 and 5). These data are consistent with those of Salbert *et al.* (36) and Soprano *et al.* (37), in which both types of retinoid receptors were required to mediate AP-1 transrepression in hepatocarcinoma cells (HepG2) and ovarian adenocarcinomas, respectively.

The anti-AP-1 activity of retinoid receptors has been considered to be one of the important mechanisms by which retinoids inhibit cell proliferation and cell transformation (14). RARs, in response to RA, could interfere with induction of gene transcription by the AP-1 protein complex (18–20). AP-1 activity can be induced by a wide variety of agents such as mitogens, phorbol esters, stress, and heat shock (20, 30, 36–38). Both insulin and EGF have been implicated in breast tumor growth and are thought to contribute to tumor malignancy (34, 35). AP-1 sites are located in the promoter region of many genes, such as collagenase, stromelysin, and ornithine decarboxylase (20, 30), which play essential roles in cancer cell proliferation and metastasis (39, 40). Inhibition of transcription of these genes may inhibit tumor development and contribute to the anticancer effect of

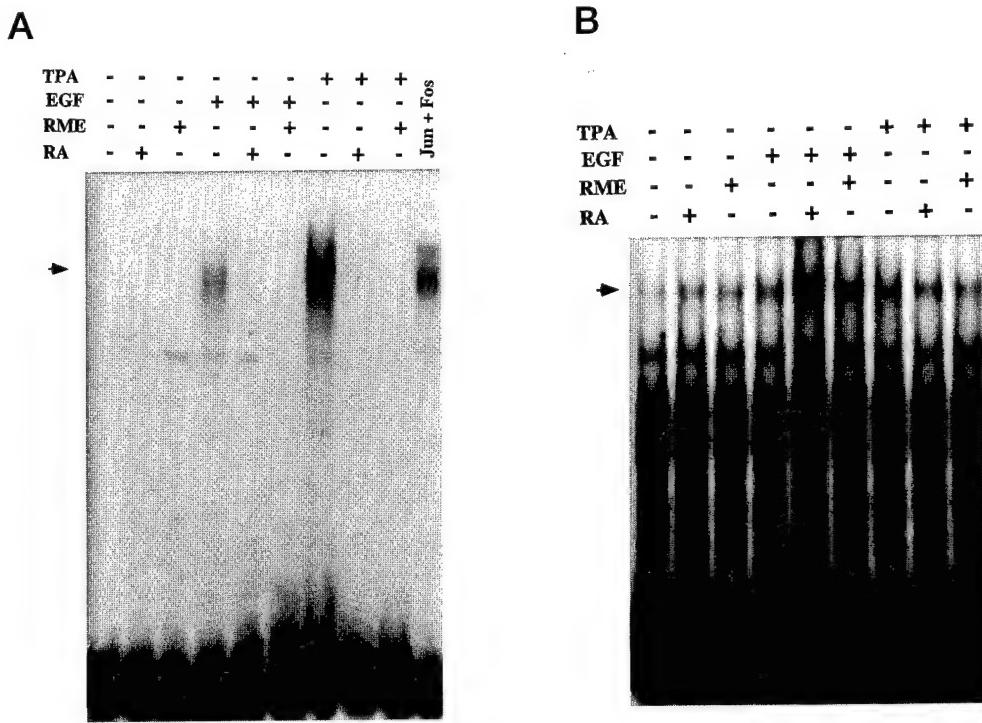


Fig. 7. Inhibition of TPA- and EGF-induced AP-1 binding activity by RME in ZR-75-1 breast cancer cells. Nuclear proteins were prepared from ZR-75-1 cells treated with TPA (100 ng/ml) or EGF (10 ng/ml) and/or RME ( $10^{-6}$  M). *A*, AP-1 binding of the nuclear proteins was analyzed by gel retardation assay using the AP-1 binding site as a probe. Arrow, AP-1 binding complex. *B*, binding of nuclear proteins to SP-1 is shown for control (arrow). The result of the gel shift shown is one of two duplicate experiments.

retinoids (14). For instance, retinoids that selectively inhibit AP-1 activity were able to inhibit the growth of lung cancer and breast cancer cells (28), and inhibit the TPA-induced transformation and clonal growth of the promotion-sensitive mouse epidermal JB6 cell line (26). Thus, the anti-AP-1 activity could contribute to the chemopreventive effect of retinoids that block the processes of tumor promotion and cell transformation. Our finding that RME can inhibit AP-1 activity induced by a variety of stimuli strongly suggests that the anti-AP-1 effect of RME may contribute to its cancer-preventive effect (5–10). To further study the mechanism by which RME downregulates transcriptional activation of AP-1, we analyzed AP-1 binding of nuclear proteins prepared from HeLa cells (Fig. 6) and ZR-75-1 cells (Fig. 7) treated with TPA or EGF that induces AP-1 activities (30, 41). Our *in vitro* data (Fig. 7) demonstrate that TPA- and EGF-induced AP-1 binding is completely inhibited when breast cancer cells are pretreated with RME, therefore providing an explanation for the *in vivo* repression of AP-1 activity observed in these cells (Figs. 4 and 5).

Modulation of retinoid receptor transactivation activity by retinoids requires their binding to the receptors. *Trans*-RA binds RARs and modulates RAR transactivation activities, whereas *9-cis*-RA binds both RARs and RXRs and activates their transcriptional function. Inhibition of AP-1 activity by retinoid receptors also requires binding of RA to the receptors (18, 19). In the present study, inhibition of AP-1 activity by RME occurred only when RAR- $\alpha$  and/or RXR- $\alpha$  were expressed (Fig. 1), suggesting that the anti-AP-1 effect of RME is also mediated by retinoid receptors. This is supported by our previous observation that RME could also influence transactivation activities of retinoid receptors on various RAREs (29). RME itself may bind to retinoid receptors and inhibit AP-1 activity *in vivo*. Alternatively, a metabolite of RME may interact with RAR or RXR, thereby modulating the anti-AP-1 activity of the receptors. Previous reports have shown that RARs repress AP-1 binding via protein-protein interactions without competition within the AP-1 binding site (18–20). Inhibition of AP-1 binding by ligand-induced retinoid receptors may be mediated by CBP that interacts with both AP-1

complex and ligand-activated nuclear receptors and acts as a common coactivator of nuclear receptors and AP-1 (22). Our DNA-binding assay data (Fig. 7) suggest that RME-induced retinoid receptors in breast cancer ZR-75-1 cells may inhibit the binding of AP-1 activity in the cells. Alternatively, RME may inhibit the expression of AP-1-binding proteins. Interestingly, RME is able to inhibit the transactivation activity of RAR- $\alpha$ , - $\beta$  and - $\gamma$ , whereas it represses AP-1 activity only in the presence of RAR- $\alpha$ , not in the presence of RAR- $\beta$  or RAR- $\gamma$ . Thus, binding of RME or its metabolite to RAR- $\beta$  or RAR- $\gamma$  may not induce the appropriate conformation required for AP-1 interaction. The finding that RME could not activate receptor transactivation (29) suggests that it represents an anti-AP-1-selective retinoid. This is consistent with the observation that RME is much less toxic than *trans*-RA *in vitro* (7). Research has been focused on the identification of retinoids that selectively inhibit AP-1 activity but do not activate the receptor transactivation function (27, 28). These anti-AP-1-selective retinoids are expected to be more effective anti-cancer agents and to have reduced side effects related to the inhibition of retinoid receptor transactivation activity.

## REFERENCES

1. Lotan, R. Effect of vitamin A and its analogs (retinoids) on normal and neoplastic cells. *Biochim. Biophys. Acta*, **605**: 33–91, 1981.
2. Gudas, L. J., Sporn, M. B., and Roberts, A. B. Cellular biology and biochemistry of the retinoids. In: M. B. Sporn, A. B. Roberts, and D. S. Goodman (eds.), *The Retinoids*, 2nd edition, pp. 443–520. New York: Raven Press, 1994.
3. Lippman, S. M., Kessler, J. F., and Meyskens, F. L. Retinoids as preventive and therapeutic anticancer agents. *Cancer Treat. Rep.*, **71**: 493–515, 1987.
4. Bollag, W., and Holdener, E. E. Retinoids in cancer prevention and therapy. *Ann. Oncol.*, **3**: 513–526, 1992.
5. Grubbs, C. J., Moon, R. C., Sporn, M. B., and Newton, D. L. Inhibition of mammary cancer by retinyl methyl ether. *Cancer Res.*, **37**: 599–602, 1977.
6. Hill, D. L., and Grubbs, C. J. Retinoids as chemopreventive and anticancer agents in intact animals. *Anticancer Res.*, **2**: 111–124, 1982.
7. Shealy, Y. F. Synthesis and evaluation of some new retinoids for cancer chemoprevention. *Prev. Med.*, **18**: 624–645, 1989.
8. Chopra, D. P., and Wilkoff, L. J. Reversal by vitamin A analogues (retinoids) of hyperplasia induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in mouse prostate organ cultures. *J. Natl. Cancer Inst.*, **58**: 923–930, 1977.
9. Mossman, B. T., Craighead, J. E., and MacPherson, B. V. Asbestos-induced epithelial

- changes in organ cultures of hamster trachea: inhibition by retinyl methyl ether. *Science* (Washington DC), **207**: 311–313, 1980.
10. Hill, D. L., and Sani, B. P. Metabolic disposition and development of new chemopreventive retinoids. *Drug Metab. Rev.*, **23**: 313–338, 1991.
  11. Mangelsdorf, D. J., and Evans, R. M. The RXR heterodimers and orphan receptors. *Cell*, **83**: 841–850, 1995.
  12. Kastner, P., Mark, M., and Chamson, P. Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell*, **83**: 859–869, 1995.
  13. Mangelsdorf, D. J., Thummel, C., Bestor, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Marle, M., Chamson, P., and Evans, R. M. The nuclear receptor superfamily: the second decade. *Cell*, **83**: 835–839, 1995.
  14. Pfahl, M. Nuclear receptor/AP-1 interaction. *Endocr. Rev.*, **14**: 651–658, 1993.
  15. Warrell, R. P., Jr., de The, H., Wang, Z.-Y., and Degos, L. Acute promyelocytic leukemia. *N. Engl. J. Med.*, **329**: 177–189, 1993.
  16. Liu, Y., Lee, M.-O., Wang, H.-G., Li, Y., Hashimoto, Y., Klaus, M., Reed, J. C., and Zhang, X.-k. RAR $\beta$  mediates the growth inhibitory effect of retinoic acid by inducing cell apoptosis in human breast cancer cells. *Mol. Cell. Biol.*, **16**: 1138–1149, 1996.
  17. Lotan, R., Xu, X.-C., Lippman, S. M., Ro, J. Y., Lee, J. J., and Hong, W. K. Suppression of retinoic acid receptor  $\beta$  in premalignant oral lesions and its up-regulation by isotretinoin. *N. Engl. J. Med.*, **332**: 1405–1410, 1995.
  18. Yang-Yen, H. F., Zhang, X.-k., Graupner, G., Tzukerman, M., Sakamoto, B., Karin, M., and Pfahl, M. Antagonism between retinoic acid receptors and AP-1: implications for tumor promotion and inflammation. *New Biol.*, **3**: 1206–1219, 1991.
  19. Schule, R., Rangarajan, P., Yang, N., Kliwera, S., Ransone, L. J., Bolaldo, J., Verma, I. M., and Evans, R. M. Retinoic acid is a negative regulator of AP-1 responsive genes. *Proc. Natl. Acad. Sci. USA*, **88**: 6092–6096, 1991.
  20. Nicholson, R., Mader, S., Nagpal, S., Leid, M., Rochette-Egly, C., and Chamson, P. Negative regulation of the rat stromelysin gene promoter by retinoic acid is mediated by an AP1 binding site. *EMBO J.*, **9**: 4443–4454, 1990.
  21. Angel, P., and Karin, M. The role of Jun, Fos, and the AP-1 complex in cell proliferation and transformation. *Biochim. Biophys. Acta*, **1072**: 129–157, 1991.
  22. Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.-C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. A CBP integrator complex that mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell*, **85**: 403–414, 1996.
  23. Chrivia, J. C., Kwok, R. P. S., Lamb, N., Hagihara, M., Montminy, M. R., and Goodman, R. H. Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature (Lond.)*, **365**: 855–859, 1993.
  24. Kwok, R. P. S., Lundblad, J. R., Chrivia, J. C., Richards, J. P., Bachinger, H. P., Brennan, R. G., Roberts, S. G., Green, M. R., and Goodman, R. H. Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature (Lond.)*, **370**: 223–226, 1994.
  25. Arias, J., Alberts, A. S., Brindle, P., Claret, F. X., Smeal, T., Karin, M., Feramisco, J., and Montimony, M. Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. *Nature (Lond.)*, **370**: 226–229, 1994.
  26. Li, J. J., Dong, Z., Dawson, M. I., and Colburn, N. H. Inhibition of tumor promoter-induced transformation by retinoids that transrepress AP-1 without transactivating retinoic acid response element. *Cancer Res.*, **56**: 483–489, 1996.
  27. Chen, J. Y., Penco, S., Ostowski, J., Balaguer, P., Pons, M., Starrett, J. E., Reczek, P., Chamson, P., and Gronemeyer, H. RAR-specific agonist/antagonists which dissociate transactivation and AP-1 transrepression inhibit anchorage-independent cell proliferation. *EMBO J.*, **14**: 1187–1197, 1995.
  28. Fanjul, A., Dawson, M. I., Hobbs, P. D., Jong, L., Cameron, J. F., Harley, E., Graupner, G., Lu, X. P., and Pfahl, M. A new class of retinoids with selective inhibition of AP-1 inhibits proliferation. *Nature (Lond.)*, **372**: 107–111, 1994.
  29. Sani, B. P., Zhang, X.-k., Hill, D. L., and Shealy, Y. F. Retinyl ethers: binding properties, chemopreventive potential, and transcriptional regulation. *Biochem. Biophys. Res. Commun.*, **223**: 293–298, 1996.
  30. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P., and Karin, M. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. *Cell*, **49**: 729–739, 1987.
  31. Luckow, B., and Schut, G. CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. *Nucleic Acids Res.*, **15**: 5490, 1987.
  32. Zhang, X.-k., Hoffmann, B., Tran, P., Graupner, G., and Pfahl, M. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. *Nature (Lond.)*, **355**: 441–446, 1992.
  33. Lee, M.-O., Liu, Y., and Zhang, X.-k. A retinoic acid response element that overlaps an estrogen response element mediates multihormonal sensitivity in transcriptional activation of the lactotrophin gene. *Mol. Cell. Biol.*, **15**: 4194–4207, 1995.
  34. Macaulay, V. M. Insulin-like growth factors and cancer. *Br. J. Cancer*, **65**: 311–320, 1992.
  35. Ozanne, B., Richars, C. S., Hendler, F., Burns, D., and Gusterson, B. Over-expression of the EGF receptor is a hallmark of squamous cell carcinomas. *J. Pathol.*, **149**: 9–14, 1986.
  36. Salbert, G., Fanjul, A., Piedrafita, F. J., Lu, X. P., Kim, S. J., Tran, P., and Pfahl, M. Retinoic acid receptors and retinoid X receptor  $\alpha$  down-regulate the transforming factor- $\beta$ 1 promoter by antagonizing AP-1 activity. *Mol. Endocrinol.*, **7**: 1347–1356, 1993.
  37. Soprano, D. R., Chen, L. X., Wu, S., Donigan, A. M., Borghaei, R. C., and Soprano, K. J. Overexpression of both RAR and RXR restores AP-1 repression in adenocarcinoma cells resistant to retinoic acid-dependent growth inhibition. *Oncogene*, **12**: 577–584, 1996.
  38. Ransone, L. J., and Verma, I. M. Nuclear proto-oncogenes Fos and Jun. *Annu. Rev. Cell Biol.*, **6**: 539–557, 1990.
  39. Lim, R. W., Zhu, C. Y., and Stringer, B. Differential regulation of primary response gene expression in skeletal muscle cell through multiple signal transduction pathways. *Biochim. Biophys. Acta*, **1266**: 91–100, 1995.
  40. Furth, L. T., Skubitz, A. P., and Fields, G. B. Tumor cell invasion, matrix metalloproteinases, and the dogma. *Lab. Invest.*, **70**: 781–783, 1994.
  41. Learman, D. W., Pisharody, S., Flickinger, T. W., Commane, M. A., Schlessinger, J., Kerr, I. M., Levy, D. E., and Stark, G. R. Roles of JAKs in activation of STATs and stimulation of c-Fos gene expression by epidermal growth factor. *Mol. Cell. Biol.*, **16**: 369–375, 1996.

## Inhibition of *trans*-Retinoic Acid-Resistant Human Breast Cancer Cell Growth by Retinoid X Receptor-Selective Retinoids

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All-*trans*-retinoic acid (*trans*-RA) and other retinoids exert anticancer effects through two types of retinoid receptors, the RA receptors (RARs) and retinoid X receptors (RXRs). Previous studies demonstrated that the growth-inhibitory effects of *trans*-RA and related retinoids are impaired in certain estrogen-independent breast cancer cell lines due to their lower levels of RAR $\alpha$  and RAR $\beta$ . In this study, we evaluated several synthetic retinoids for their ability to induce growth inhibition and apoptosis in both *trans*-RA-sensitive and *trans*-RA-resistant breast cancer cell lines. Our results demonstrate that RXR-selective retinoids, particularly in combination with RAR-selective retinoids, could significantly induce RAR $\beta$ , and inhibit the growth and induce the apoptosis of *trans*-RA-resistant, RAR $\alpha$ -deficient MDA-MB-231 cells, but had low activity against *trans*-RA-sensitive ZR-75-1 cells that express high levels of RAR $\alpha$ . Using gel retardation and transient transfection assays, we found that the effects of RXR-selective retinoids on MDA-MB-231 cells were most likely mediated by RXR-nur77 heterodimers that bound to the RA response element in the RAR $\beta$  promoter and activated the RAR $\beta$  promoter in response to RXR-selective retinoids. In contrast, growth inhibition by RAR-selective retinoids in *trans*-RA-sensitive, RAR $\alpha$ -expressing cells most probably occurred through RXR-RAR $\alpha$  heterodimers that also bound to and activated the RAR $\beta$  promoter. In MDA-MB-231 clones stably expressing RAR $\alpha$ , both RAR $\beta$  induction and growth inhibition by RXR-selective retinoids were suppressed, while the effects of RAR-selective retinoids were enhanced. Together, our results demonstrate that activation of RXR can inhibit the growth of *trans*-RA-resistant MDA-MB-231 breast cancer cells and suggest that low cellular RAR $\alpha$  may regulate the signaling switch from RAR-mediated to RXR-mediated growth inhibition in breast cancer cells.

Retinoids, the natural and synthetic vitamin A analogs, exert profound effects on cell proliferation, differentiation, and apoptosis (19, 36, 50) and are considered promising agents for the prevention and treatment of human cancers, including breast cancer (36, 43, 50). Retinoids, alone or in combination with an antiestrogen or interferons, inhibit the *in vitro* growth of human breast cancer cells (12–14, 29, 34, 51, 60–62). The natural retinoid derivative retinyl methyl ether (18) and the synthetic retinoids *N*-(4-hydroxyphenyl) retinamide (4-HPR) (44, 45) and LGD1069 (17) effectively inhibited the development of carcinogen-induced mammary cancers in animals. Unfortunately, clinical trials on patients with advanced breast cancer showed no significant activity for retinoids (2, 3, 42). These studies indicate that retinoids are effective inhibitors of the cancer cells at the early stages of tumor progression and that their effectiveness diminishes as cells become more malignant and invasive. They are also consistent with well-documented *in vitro* observations that growth inhibition by all-*trans*-retinoic acid (*trans*-RA) and related retinoids occurs mainly in estrogen-dependent, estrogen receptor-positive breast cancer cells and that upon progression to estrogen independence and loss of the estrogen receptor, most breast cancer cells become refractory to growth inhibition by *trans*-RA (14, 34, 54, 55, 60).

The effects of retinoids are mainly mediated by two classes of nuclear receptors, the RA receptors (RARs) and retinoid X receptors (RXRs) (28, 38, 70). 9-*cis*-RA is a high-affinity natural ligand for both RARs and RXRs, whereas *trans*-RA is a

high-affinity natural ligand only for the RARs. RARs and RXRs are each encoded by three distinct genes ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) and are members of the steroid/thyroid hormone/retinoid receptor superfamily, which function as ligand-activated transcription factors (28, 38, 70). RARs interact with RXRs, forming RXR/RAR heterodimers that bind to RA response elements (RAREs) to control the expression of RA-responsive genes in the presence of retinoids. Transcriptional regulation of RA-responsive genes is also modulated by a number of cofactors that appear to provide a direct link to the core transcriptional machinery and/or to modulate chromatin structure (reference 27 and references therein). Although RXR acts as a silent heterodimerization partner of RAR in CV-1 cells (15, 30, 38), recent studies demonstrate that binding of certain RXR ligands contributes to activation of RXR-RAR heterodimers in some cell types (4, 31, 40, 52, 59, 71). In the presence of 9-*cis*-RA, RXRs can also function as homodimers that bind a set of specific DNA sequences (68, 70, 71). Furthermore, activation of RXR is required for the function of other RXR-containing heterodimers, such as RXR-nur77 (15, 49) and RXR-LXR (63). Thus, distinct retinoid signaling pathways through activation of either RAR or RXR exist; however, the role of RXR activation in these pathways requires clarification.

RA target genes, including those for the RARs, have been identified. The RARE ( $\beta$ RARE) in the RAR $\beta$  gene promoter mediates *trans*-RA-induced RAR $\beta$  gene expression in many different cell types (10, 21, 57) and binds both RXR-RAR (28, 38, 70) and RXR-nur77 (15, 49) heterodimers. Gene transcriptional activation by RXR-RAR binding is mainly activated by RAR-specific ligands, while transactivation by RXR-nur77 is induced by RXR-specific ligands (15, 49). These observations

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suggest that RAR $\beta$  can be induced by both RAR- and RXR-specific ligands. nur77 is an orphan member of nuclear receptor superfamily that regulates gene expression by binding to the nur77-binding response element (NBRE) as a monomer (64). It is rapidly induced by various stimuli, including growth factors and phorbol ester- and cyclic AMP-dependent synthesis pathways (20, 39). Recent studies suggest that nur77 is involved in activation-induced apoptosis of T cells (35, 65) and is associated with *trans*-RA resistance in human lung cancer cells (66). Thus, RAR $\beta$  expression is regulated by growth signals and may be associated with the apoptotic process.

Recently, evidence has emerged that the absence or aberrant expression of RAR $\beta$  correlates with malignancy and may contribute to the development of cancers. The involvement of RAR $\beta$  in cancer development was originally implicated in the finding that the RAR $\beta$  gene is integrated by hepatitis B virus in human liver cancer (9). Subsequent reports indicate that abnormal expression of the RAR $\beta$  gene appears to be involved in the tumorigenicity of human papillomavirus type 18-transformed ovarian cancer cells (1) and the neoplastic progression of human oral squamous cell carcinoma cell lines (24), and it is observed in many other human cancer cell lines (16, 22, 24, 34, 48, 58, 69). RAR $\beta$  also suppresses the growth of breast cancer cells (33, 34, 58) and lung cancer cells (23). The auto-induction of RAR $\beta$  gene expression presumably plays a critical role in amplifying retinoid responses and is associated with the growth-inhibitory effects of *trans*-RA in breast cancer cells *in vitro* (34) and the clinical response to retinoids in patients with premalignant oral lesions (37). Retinoids that fail to induce RAR $\beta$  expression cannot arrest the growth of melanoma cells (5).

The involvement of retinoid receptors in mediating retinoid-induced growth inhibition and apoptosis has been investigated. Several studies demonstrated that expression of RAR $\alpha$  mediates the growth-inhibitory effect of *trans*-RA in estrogen-dependent breast cancer cells and that the loss of *trans*-RA sensitivity in estrogen-independent cells may be due to low levels of RAR $\alpha$  (34, 51, 55, 60). RAR $\alpha$  levels are higher in certain estrogen-dependent, *trans*-RA-sensitive breast cancer cell lines, such as ZR-75-1, MCF-7, and T-47D, than in certain estrogen-independent, *trans*-RA-resistant cell lines, such as MDA-MB-231 and MDA-MB-468 (34, 51, 55, 60). Expression of RAR $\alpha$  in estrogen-independent, *trans*-RA-resistant MDA-MB-231 breast cancer cells restored *trans*-RA sensitivity (34, 54, 55, 61). Growth inhibition induced by retinoids in estrogen-dependent MCF-7 breast cancer cells correlated with their binding affinity to RAR $\alpha$  (7). The involvement of RAR $\beta$  was suggested by the observation that it was expressed in response to *trans*-RA in certain estrogen-dependent, *trans*-RA-sensitive breast cancer cell lines, such as ZR-75-1 and T-47D, but not in estrogen-independent, *trans*-RA-resistant cell lines MDA-MB-231, MDA-MB-468, and BT-20 (34). In addition, we (34) and others (33, 53) demonstrated that introduction of RAR $\beta$  into MDA-MB-231 cells led to the recovery of *trans*-RA-induced growth inhibition. Moreover, RAR $\beta$  expression was enhanced in RAR $\alpha$  stably transfected MDA-MB-231 cells, a finding that suggests that RAR $\beta$  may mediate the growth inhibitory effects of RAR $\alpha$  (34). The role of RAR $\beta$  in growth inhibition is also supported by the observation that as normal human mammary epithelial cells senesce, RAR $\beta$  mRNA expression increases (58). RAR $\gamma$  is highly expressed in various breast cancer cell lines independently of their estrogen responsiveness (34) and is unlikely involved in regulating *trans*-RA-induced growth inhibition and apoptosis. However, recent studies (11, 12) have demonstrated that it may play a role in mediating growth inhibition and apoptosis induction by 4-HPR and certain syn-

thetic retinoids. 4-HPR was a potent transactivator of RAR $\gamma$  at concentrations that inhibited the growth and induced apoptosis of breast cancer cells (11). Furthermore, growth inhibition by certain receptor-selective retinoids and interferons was associated with increased expression of RAR $\gamma$  (12). Thus, different retinoid receptors, which may function through different mechanisms, can mediate growth inhibition and apoptosis induction by different types of retinoids in breast cancer cells.

In this study, we evaluated the effects of RAR- and RXR-class selective retinoids on the growth of *trans*-RA-resistant, RAR $\alpha$ -deficient MDA-MB-231 cells. Our results demonstrate that RXR-selective retinoids induced RAR $\beta$  expression, growth inhibition, and apoptosis in these cells, most likely through their activation of RXR-nur77 heterodimers that bind to the RAR $\beta$  promoter. When we stably expressed RAR $\alpha$  in MDA-MB-231 cells, we observed an enhanced growth inhibition and RAR $\beta$  induction by RAR-selective retinoids and decreased effects by RXR-selective retinoids, similar to those observed in *trans*-RA-sensitive, RAR $\alpha$ -expressing breast cancer cells, such as ZR-75-1 cells. Thus, a RXR-mediated growth inhibition pathway exists in breast cancer cells and is regulated by RAR $\alpha$  levels. These results may provide a novel method for inhibiting the growth of the more malignant *trans*-RA-resistant breast cancer cells.

#### MATERIALS AND METHODS

**Retinoids.** *trans*-RA was obtained from Sigma (St. Louis, Mo.), SR11246, SR11237, and SR11247 were prepared as described by Dawson et al. (8).

(E)-3-[4-(1-Methoxy-5,6,7,8-tetrahydro-1,5,5,8,8-tetramethyl-3-naphthalenyl)phenyl]propenoic acid (SR11278) was synthesized as follows. (i) Cyclization [AlCl<sub>3</sub>, (CH<sub>2</sub>Cl)<sub>2</sub>, 0°C] of 3-bromoanisole with 2,5-dichloro-2,5-dimethylhexane as reported by Kagechika et al. (26) yielded 3-bromo-1-methoxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene (71%), which was coupled under Suzuki conditions {Pd[P(C<sub>6</sub>H<sub>5</sub>)<sub>3</sub>]<sub>4</sub>, NaHCO<sub>3</sub>, aqueous MeO(CH<sub>2</sub>)<sub>2</sub>OMe, reflux} (41) with 4-formylphenylboronic acid to give 4-(1-methoxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-3-naphthalenyl)benzaldehyde (91%). (ii) Horner-Emmons olefination of this benzaldehyde with triethyl phosphonoacetate [KN(SiMe<sub>3</sub>)<sub>2</sub>, tetrahydrofuran-toluene, -78 to 25°C] produced the ethyl ester of SR11278 (97%). (iii) Hydrolysis (KOH, aqueous ethanol/EtOH; aqueous HCl) gave SR11278 (99%): melting point (mp), 179 to 182°C; <sup>1</sup>H nuclear magnetic resonance (NMR) (300 MHz, <sup>2</sup>HCCl<sub>3</sub>) δ 1.34 (s, 6, CMe<sub>2</sub>), 1.41 (s, 6, CMe<sub>2</sub>), 1.68 (m, 4, CH<sub>2</sub>CH<sub>2</sub>), 3.89 (s, 3, OMe), 6.49 (d, J = 16.0 Hz, 1, C=CHCO<sub>2</sub>), 6.90 (d, J = 1.8 Hz, 1, ArH), 7.20 (d, J = 1.8 Hz, 1, ArH), 7.62 (s, 4, ArH), 7.83 (d, J = 16.0 Hz, 1, HC=CCO<sub>2</sub>); IR (KBr) 2,955, 1,700, 1,630, 1,430, 1,278, 1,220, 825 cm<sup>-1</sup>.

4-[3-(3-Hydroxy-5,6,7,8-tetrahydro-3,5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido]benzoic acid (SR11281) was synthesized as follows. (i) Fries rearrangement [AlCl<sub>3</sub>, 130°C (23)] of 2-acetoxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene produced 1-(3-hydroxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)ethanol (90%). (ii) Protection of the phenolic group as the benzyl ether (benzyl bromide, K<sub>2</sub>CO<sub>3</sub>, acetone, reflux; 92%) and oxidation of the acetyl group (NaOCl, EtOH, reflux) gave 3-benzoyloxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-carboxylic acid (30%). (iii) The carboxylic acid was converted (oxalyl chloride, CH<sub>2</sub>Cl<sub>2</sub>) to the acyl chloride and treated (pyridine-benzene) with ethyl 4-aminobenzoate to yield the benzamide (95%). (iv) Ester hydrolysis (NaOH, aqueous EtOH, 25°C; aqueous HCl; 97%) and hydrogenolysis [H<sub>2</sub>, Pd(C), EtOH, 25°C; 93%] of the benzyl ether protecting group afforded SR11281: mp, 275 to 278°C; <sup>1</sup>H NMR (300 MHz, <sup>2</sup>HCCl<sub>3</sub>) δ 1.29 (s, 6, CMe<sub>2</sub>), 1.32 (s, 6, CMe<sub>2</sub>), 1.69 (s, 4, CH<sub>2</sub>CH<sub>2</sub>), 6.93 (s, 1, ArH), 7.69 (s, 1, ArH), 7.74 (d, 2, J = 8.8 Hz, ArH), 8.08 (d, 2, J = 8.8 Hz, ArH); IR (KBr) 3,330, 1,686, 1,530, 1,419, 1,174 cm<sup>-1</sup>.

4-[1-(5,6,7,8-Tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenyl)-2-methylpropenyl]benzoic acid (SR11345) was prepared by Suzuki Pd(0)-catalyzed coupling {Pd[P(C<sub>6</sub>H<sub>5</sub>)<sub>3</sub>]<sub>4</sub>, NaHCO<sub>3</sub>, aqueous MeO(CH<sub>2</sub>)<sub>2</sub>OMe, reflux; 58%} (8) between 5,6,7,8-tetrahydro-3,5,5,8,8-pentamethylnaphthalene-2-boronic acid and methyl (1-bromo-2-methylpropenyl)benzoate and hydrolysis (KOH, aqueous EtOH; aqueous HCl; 91%). The first intermediate was synthesized in two steps by bromination (Br<sub>2</sub>, CHCl<sub>3</sub>, 25°C; 80%) of 5,6,7,8-tetrahydro-3,5,5,8,8-pentamethylnaphthalene, followed by conversion to the arylboronic acid [*n*-BuLi, THF, -78°C to ambient temperature; B(OMe)<sub>3</sub>, -78°C to ambient temperature; aqueous NH<sub>4</sub>Cl; 86%]. The second intermediate was obtained from methyl (2-methylpropenyl)benzoate by bromination (Br<sub>2</sub>, CH<sub>2</sub>Cl<sub>2</sub>; 100%) and dehydrobromination [1,8-diazabicyclo[5.4.0]undec-7-ene, MeO(CH<sub>2</sub>)<sub>2</sub>OMe, 25°C; 90%] to yield SR11345: mp, 246 to 247°C; <sup>1</sup>H NMR (<sup>2</sup>HCCl<sub>3</sub>) δ 1.25 (s, 12, 4CH<sub>3</sub>), 1.64 (s, 3, CH<sub>3</sub>), 1.66 (s, 3, CH<sub>3</sub>), 1.88 (s, 4, (CH<sub>2</sub>)<sub>2</sub>), 1.99 (s, 3, CH<sub>3</sub>), 7.00 (s, 1, ArH), 7.03 (s, 1, ArH), 7.25 (d, J = 8.3, 2, ArH), 7.98 (d, J = 8.3, 2, ArH); IR (KBr)

3,500 to 2,300 (OH), 1,687 (C = O), 1,606 (C = C)  $\text{cm}^{-1}$ ; EI-HRMS ( $\text{NH}_3^+$ ) calculated for  $\text{C}_{26}\text{H}_{32}\text{O}_2 + \text{NH}_4^+$ , 394.2746; found, 394.2751.

4-(1-Amino-5,6,7,8-tetrahydro-5,5,8,8-tetramethylanthracen-2-yl)benzoic acid (SR11350) was synthesized by nitration ( $\text{HNO}_3$ ,  $\text{Ac}_2\text{O}-\text{HOAc}$ ,  $-10^\circ\text{C}$ ) of 6-bromo-1,2,3,4-tetrahydro-1,1,4,4-tetramethylanthracene to give after chromatographic separation (silica,  $\text{CH}_2\text{Cl}_2$ -hexanes) of isomers 6-bromo-1,2,3,4-tetrahydro-1,1,4,4-tetramethyl-5-nitroanthracene (26%), which was coupled {Pd[P(C<sub>6</sub>H<sub>5</sub>)<sub>3</sub>]<sub>4</sub>,  $\text{NaHCO}_3$ , aqueous DME,  $80^\circ\text{C}$ ; 83%} to 4-carbethoxyphenylboronic acid to afford ethyl 4-(1-nitro-5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-anthracen-2-yl)benzoate. Hydrogenation [ $\text{H}_2$ , Pd(C),  $\text{EtOAc}$ ,  $25^\circ\text{C}$ ; 95%] to the amine and ester hydrolysis (KOH, aqueous EtOH;  $85^\circ\text{C}$ ; aqueous HCl; 93%) yielded SR11350 as the HCl salt mp, 267 to 268°C; <sup>1</sup>H NMR (300 MHz,  $\text{Me}_2\text{SO}-\text{D}_2\text{H}_6$ ) δ 1.40 (s, 6,  $\text{CH}_3$ ), 1.44 (s, 6,  $\text{CH}_3$ ), 1.77 (s, 4,  $\text{CH}_2$ ), 7.25 (d,  $J = 8.6$  Hz, 1, ArH) 7.55 (d,  $J = 8.6$  Hz, 1, ArH), 7.55 (d,  $J = 8.2$  Hz, 2, ArH), 7.90 (s, 1, ArH), 8.11 (d,  $J = 8.2$  Hz, 2, ArH), 8.21 (s, 1, ArH); EI-HRMS calculated for  $\text{C}_{25}\text{H}_{27}\text{NO}_2$ , 373.2042; found, 373.2040.

6-[3-(1-Adamantyl)-5-methoxyphenyl]naphthalene-2-carboxylic acid (SR11362) was obtained by hydrolysis (KOH, aqueous EtOH,  $85^\circ\text{C}$ ; aqueous HCl; 90%) of its ethyl ester; mp, 236 to 238°C; <sup>1</sup>H NMR (300 MHz, <sup>2</sup>HCCl<sub>3</sub>) δ 1.79 (m, 6,  $\text{CH}_2$ ), 1.98 (m, 6,  $\text{CH}_2$ ), 2.12 (m, 3, CH), 3.87 (s, 3,  $\text{OCH}_3$ ), 6.96 (s, 1, ArH), 7.02 (s, 1, ArH), 7.28 (s, 1, ArH), 7.75 (m, 1, ArH), 7.96 (m, 3, ArH), 8.12 (d,  $J = 8$  Hz, 1, ArH), 8.68 (s, 1, ArH); EI-HRMS calculated for  $\text{C}_{28}\text{H}_{28}\text{O}_3$ , 412.2038; found, 412.2030.

2-(4-Carboxyphenyl)-6,7,8,9-tetrahydro-6,6,9,9-tetramethylbenzo[g]quinoline-1-oxide (SR11365) was prepared by acetylation ( $\text{Ac}_2\text{O}$ ,  $\text{Et}_3\text{N}$ ,  $\text{EtOAc}$ ,  $25^\circ\text{C}$ ; 100%) of 5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene-2-amine to the amide, which on reaction with excess Vilsmeier reagent {[POC]<sub>3</sub>, 8.5 equivalents; alerts DMF 3.5 equivalents; alerts (CH<sub>2</sub>Cl)<sub>2</sub>,  $0^\circ\text{C}$ ; 25 to  $85^\circ\text{C}$ ; aqueous  $\text{NaHCO}_3$ ; 61%} produced 2-chloro-6,7,8,9-tetrahydro-6,6,9,9-tetramethylbenzo[g]quinoline-3-carboxaldehyde, which on coupling {Pd[P(C<sub>6</sub>H<sub>5</sub>)<sub>3</sub>]<sub>4</sub>,  $\text{NaHCO}_3$ , aqueous DME,  $80^\circ\text{C}$ ; 71%} with 4-carbethoxyphenylboronic acid yielded ethyl 4-[3-formyl-6,7,8,9-tetrahydro-6,6,9,9-tetramethylbenzo[g]quinolin-2-yl]benzoate. Oxidation of the formyl group to the carboxylic acid with concomitant base hydrolysis of the ester ( $\text{Ag}_2\text{O}$ , NaOH, aqueous EtOH-THF,  $25^\circ\text{C}$ ;  $\text{H}_2\text{O}^+$ ; 100%) gave the dicarboxylic acid, which on thermal decarboxylation ( $315^\circ\text{C}$ ; 93%) yielded 4-[6,7,8,9-tetrahydro-6,6,9,9-tetramethylbenzo[(100g)quinolin-2-yl]benzoic acid, which has also been synthesized by another route (26). Esterification ( $\text{SOCl}_2$ , reflux;  $\text{MeOH}-\text{C}_5\text{H}_5\text{N}-\text{C}_6\text{H}_5\text{CH}_3$ , aqueous  $\text{NaHCO}_3$ ; 91%), *N*-oxidation ( $3-\text{ClC}_6\text{H}_4\text{CO}_2\text{H}$ ,  $\text{CHCl}_3$ ,  $25^\circ\text{C}$ ; 85%), and ester hydrolysis (NaOH, aqueous EtOH,  $70^\circ\text{C}$ ; aqueous citric acid; 72%) yielded SR11365: mp, >300°C; <sup>1</sup>H NMR (300 MHz,  $\text{Me}_2\text{SO}-\text{D}_2\text{H}_6$ ) δ 1.42 (s, 12,  $\text{CH}_3$ ), 1.80 (s, 4,  $\text{CH}_2$ ), 7.68 (d,  $J = 9$  Hz, 1, ArH), 7.95 (d,  $J = 9$  Hz, 1, ArH), 8.10 (d,  $J = 8$  Hz, 2, ArH), 8.14 (s, 1, ArH), 8.15 (d,  $J = 8$  Hz, 2H, ArH), 8.63 (s, 1, ArH); EI-HRMS calculated for  $\text{C}_{24}\text{H}_{25}\text{NO}_3$ , 375.1834; found, 375.1832.

**Gel culture.** Breast cancer cell lines ZR-75-1, T-47D, and MDA-MB-231 were obtained from the American Type Culture Collection. ZR-75-1 and T-47D cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, and MDA-MB-231 and CV-1 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

**Growth inhibition assay.** To study anchorage-dependent growth inhibition, cells were seeded at 500 cells per well in 96-well plates and treated with solvent control (dimethyl sulfoxide-EtOH) or with  $10^{-6}$  M retinoids (or  $10^{-7}$  M trans-RA) in solvent. Media were changed every 48 h. After treatment for 10 days, the number of viable cells were determined by their capacity to convert the tetrazolium salt MTT into a blue formazan product, using a cell proliferation-cytotoxicity assay kit (Promega, Madison, Wis.) (46).

**RNA preparation and Northern blot.** For Northern blot analysis, total RNAs were prepared by the guanidine hydrochloride-ultracentrifugation method (34). About 30- $\mu\text{g}$  aliquots of total RNAs from different cell lines treated with or without  $10^{-6}$  M retinoids (or  $10^{-7}$  M trans-RA) were fractionated on 1% agarose gel, transferred to nylon filters, and probed with the <sup>32</sup>P-labeled ligand-binding domain of receptor cDNAs as previously described (34). To determine that equal amounts of RNA were used, the filters were also probed with  $\beta$ -actin.

**Plasmids, receptor proteins, and nuclear extract preparation.** The nur77 expression vector was constructed by cloning the nur77 cDNA fragment into pECE or pBluescript vector as described previously (66). The construction of the chloramphenicol acetyltransferase (CAT) reporter containing the RAR $\beta$  promoter (*Bgl*II-*Bam*HI fragment) and expression vectors for RAR $\alpha$ , RAR $\beta$ , and RXR $\alpha$  have been described elsewhere (21, 67, 68). The RXR $\alpha$  N-terminal deletion mutant ( $\Delta$ RXR10) was constructed by deleting 61 amino acid residues from its N-terminal end as described previously (71). Receptor proteins were synthesized by an in vitro transcription-translation system using rabbit reticulocyte lysate (Promega) as described previously (67). The relative amount of the translated proteins was determined by [<sup>35</sup>S]-methionine-labeled protein on sodium dodecyl sulfate-polyacrylamide gels by quantitating and then normalizing the amount of incorporated radioactivity relative to the content of methionine in each protein. Nuclear extracts were prepared as previously described (34).

**Gel retardation assay.** The gel retardation assay using in vitro-synthesized proteins or nuclear extracts has been described previously (67, 68). When antibodies were used, 1  $\mu\text{l}$  of anti-nur77 (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) or 1  $\mu\text{l}$  of anti-RXR (32) was incubated with receptor protein at room temperature for 30 min prior to the assay.

TABLE 1. Retinoid transcriptional activation activity

Retinoid	Relative receptor transactivation (%) <sup>a</sup>			
	RAR $\alpha$	RAR $\beta$	RAR $\gamma$	RXR $\alpha$
trans-RA	100	100	100	53
9-cis-RA	122	128	137	100
SR11246	6	12	9	98
SR11277	5	149	65	0
SR11278	13	145	67	0
SR11281	59	123	133	0
SR11284	0	25	45	0
SR11345	0	-6	-12	107
SR11365	95	56	48	6
SR11371	2	20	3	0
SR11383	66	71	16	19
SR11384	33	27	0	7

<sup>a</sup> Transcriptional activation in CV-1 cells, using the (TREpal)<sub>2</sub>-tk-CAT reporter, compared to 1  $\mu\text{M}$  trans-RA for RARs or 1  $\mu\text{M}$  9-cis-RA for RXR $\alpha$  as 100%.

**Transient transfection and CAT assay.** To measure transcriptional activation of the RAR $\beta$  promoter by retinoids, this promoter (*Bgl*II-*Bam*HI fragment) was linked to the CAT gene reporter (21). The reporter plasmid and  $\beta$ -galactosidase expression vector (pCH110; Pharmacia) (100 ng each) with or without the RAR $\beta$  expression vector were transiently transfected into CV-1 cells by the calcium phosphate precipitation method (34). Cells were grown in the presence or absence of  $10^{-6}$  M retinoids or  $10^{-7}$  M trans-RA. Transfection efficiency was normalized to  $\beta$ -galactosidase activity. The data shown are the means of three separate experiments.

**Stable transfection.** RAR $\alpha$  cDNA was cloned into the pRc/CMV expression vector (Invitrogen, San Diego, Calif.) as described elsewhere (34). The resulting recombinant constructs were then stably transfected into MDA-MB-231 breast cancer cells by the calcium phosphate precipitation method and screened by using G418 (Gibco BRL, Grand Island, N.Y.). The levels of exogenous RAR $\alpha$  expression were determined by Northern blotting.

**Apoptosis analysis.** Cells were treated with or without  $10^{-6}$  M retinoids (or  $10^{-7}$  M trans-RA). After 48 h, cells were trypsinized, washed with phosphate-buffered saline (PBS; pH 7.4), and fixed in 1% formaldehyde in PBS. After washing with PBS, cells were resuspended in 70% ice-cold EtOH and immediately stored at  $-20^\circ\text{C}$  overnight. Cells were then labeled with biotin-16-dUTP by terminal deoxynucleotidyltransferase (TdT) and stained with avidin-fluorescein isothiocyanate (Boehringer, Mannheim, Germany). Fluorescently labeled cells were analyzed using a FACScaler-Plus as described previously (34). Representative histograms are shown.

## RESULTS

**RXR-selective retinoids inhibit the growth and induce the apoptosis of trans-RA-resistant MDA-MB-231 but not trans-RA-sensitive ZR-75-1 cells.** Because trans-RA effectively inhibited the growth and induced the apoptosis of trans-RA-sensitive, estrogen-dependent ZR-75-1 breast cancer cells, whereas it had little effect on trans-RA-resistant, estrogen-independent MDA-MB-231 breast cancer cells (34), RAR- and RXR-class selective retinoids (Table 1) were evaluated for the ability to inhibit the growth and induce the apoptosis of these cell lines. At  $10^{-6}$  M, SR11278, SR11281, SR11350, SR11362, and SR11365 activated only the RARs, not RXR $\alpha$ , on the (TREpal)<sub>2</sub>-tk-CAT reporter construct (67, 68), as determined by transient transfection in CV-1 cells. SR11237, SR11246, and SR11247 activated both RXR $\alpha$  and RAR $\beta$ , whereas SR11345 activated only RXR $\alpha$  (Table 1). Both breast cancer cell lines were treated for 10 days with  $10^{-6}$  M the indicated class-selective retinoid alone or the combination of RXR-selective SR11345 and a RAR-selective retinoid. Cell viability was determined by the MTT assay. As shown in Fig. 1, the RAR-selective retinoids strongly inhibited ZR-75-1 cell growth (55 to 75%). In contrast, the RXR-selective retinoids were far less effective inhibitors (8 to 25%) of growth. ZR-75-1 cell growth inhibition by any of the RAR-selective retinoids was only

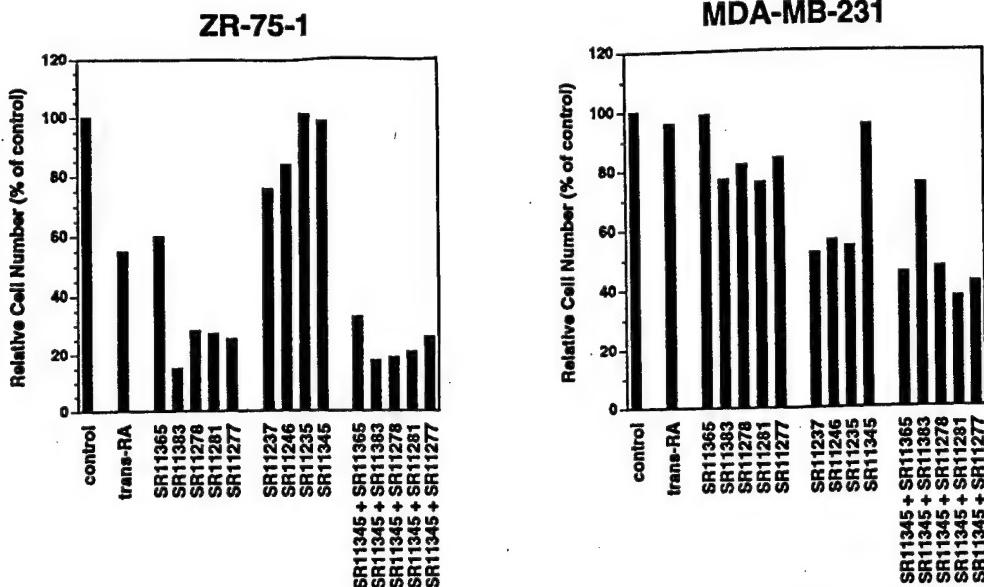


FIG. 1. Growth-inhibitory effects of retinoids on *trans*-RA-sensitive, estrogen-dependent ZR-75-1 and *trans*-RA-resistant, estrogen-independent MDA-MB-231 breast cancer cells. Cells (800 cells/well) were seeded in 96-well plates and treated with the indicated retinoids ( $10^{-6}$  M) or *trans*-RA ( $10^{-7}$  M) alone or in combination for 10 days. The number of viable cells was determined by the MTT assay.

slightly enhanced by the RXR-selective SR11345. Therefore, growth inhibition of *trans*-RA-sensitive ZR-75-1 cells by retinoids is mediated mainly by the RAR pathway, not the RXR pathway, a finding which is consistent with previous observations (34, 55). Interestingly, in MDA-MB-231 cells, RXR-selective SR11237, SR11246, or SR11247 at  $10^{-6}$  M inhibited growth (45 to 50%) more effectively than any RAR-selective retinoid, which was a poor inhibitor (<20%) (Fig. 1). The more RXR-specific retinoid SR11345 was less effective, with only 8% inhibition. However, when it was used together with one of the RAR-selective retinoids, growth inhibition was increased to 40 to 55%. These results indicate that activation of both RAR and RXR signaling pathways is required for effective cancer cell growth inhibition.

We next investigated the apoptosis-inducing effects of RAR-selective SR11365 and of *trans*-RA- and RXR-selective SR11246 in ZR-75-1 and MDA-MB-231 cells by the TdT assay (Fig. 2). *trans*-RA at  $10^{-7}$  M and SR11365 at  $10^{-6}$  M significantly induced apoptosis of ZR-75-1 cells, producing 58 and 27% apoptosis, respectively, whereas RXR-selective SR11246 produced only about 5% apoptotic cells (Fig. 2A). About 44% of MDA-MB-231 cells underwent apoptosis on treatment with SR11246, but apoptosis was not significant on treatment with *trans*-RA (4%) or SR11365 (6%) (Fig. 2B). Together, these results demonstrated that RXR-selective retinoids can induce growth inhibition and apoptosis of *trans*-RA-resistant MDA-MB-231 cells, whereas RAR-selective retinoids are more effective in *trans*-RA-sensitive cells.

**Induction of RAR $\beta$  in *trans*-RA-resistant breast cancer cells by RXR-selective retinoids.** We previously demonstrated that RAR $\beta$  expression levels in breast cancer cells correlated with the extent of growth inhibition and apoptosis induction by *trans*-RA (34). To determine whether growth inhibition and apoptosis induction by RXR-selective retinoids in *trans*-RA-resistant MDA-MB-231 cells were also associated with their induction of RAR $\beta$ , we compared the effect of RXR-selective retinoids SR11246 and SR11345 and RAR-selective SR11365, as well as the SR11345 and SR11365 combination, on RAR $\beta$

expression in these cells (Fig. 3). For comparison, *trans*-RA-sensitive ZR-75-1 and T-47D cells were studied. Both *trans*-RA and RAR-selective SR11365, but not RXR-selective SR11246 or SR11345, induced RAR $\beta$  expression in ZR-75-1 and T-47D cells. However, RXR-selective SR11246 and SR11345 induced RAR $\beta$  expression in *trans*-RA-resistant MDA-MB-231 cells at a level comparable to that observed with *trans*-RA or SR11365. A further induction of RAR $\beta$  was observed when MDA-MB-231 cells were treated with both RXR-selective SR11345 and RAR-selective SR11365. These results demonstrate that activation of RXR by RXR-selective retinoids induced RAR $\beta$  in *trans*-RA-resistant MDA-MB-231 cells, while these retinoids were unable to activate the RXR-pathway for inducing RAR $\beta$  in *trans*-RA-sensitive ZR75-1 and T-47D breast cancer cells. Thus, induction of RAR $\beta$  by RXR-selective retinoids may contribute to the effects of these retinoids on growth inhibition and apoptosis induction in *trans*-RA-resistant MDA-MB-231 cells.

**RXR-selective retinoids activate the RAR $\beta$  promoter through RXR/nur77 heterodimers.** RXR ligands can regulate gene expression through RXR homodimers (68) or certain RXR heterodimers, such as RXR-RAR (4, 31), RXR-LXR (63), or RXR-nur77 (15, 49). Regulation of gene expression by RXR-nur77 heterodimers occurs through their binding to DR-5 type RAREs (15, 49). Because the  $\beta$ RARE in the RAR $\beta$  promoter is a DR-5 type RARE and contains an NBRE (15, 49, 64), we investigated whether induction of RAR $\beta$  expression in MDA-MB-231 cells (Fig. 3) could be due to apparent activation of RXR-nur77 heterodimers on the  $\beta$ RARE by RXR-selective retinoids. nur77 alone did not show any clear binding to the  $\beta$ RARE under our experimental conditions but in the presence of RXR produced a strong complex, whose binding was largely affected by either anti-RXR or anti-nur77 antibody (Fig. 4A). For a better distinction between RXR-RAR and RXR-nur77 heterodimers, an RXR $\alpha$  mutant deleted of 68 amino acid residues from its N-terminal end (71) was used. The deletion did not affect heterodimerization properties of the RXR with RAR or nur77 (data not shown). The binding of

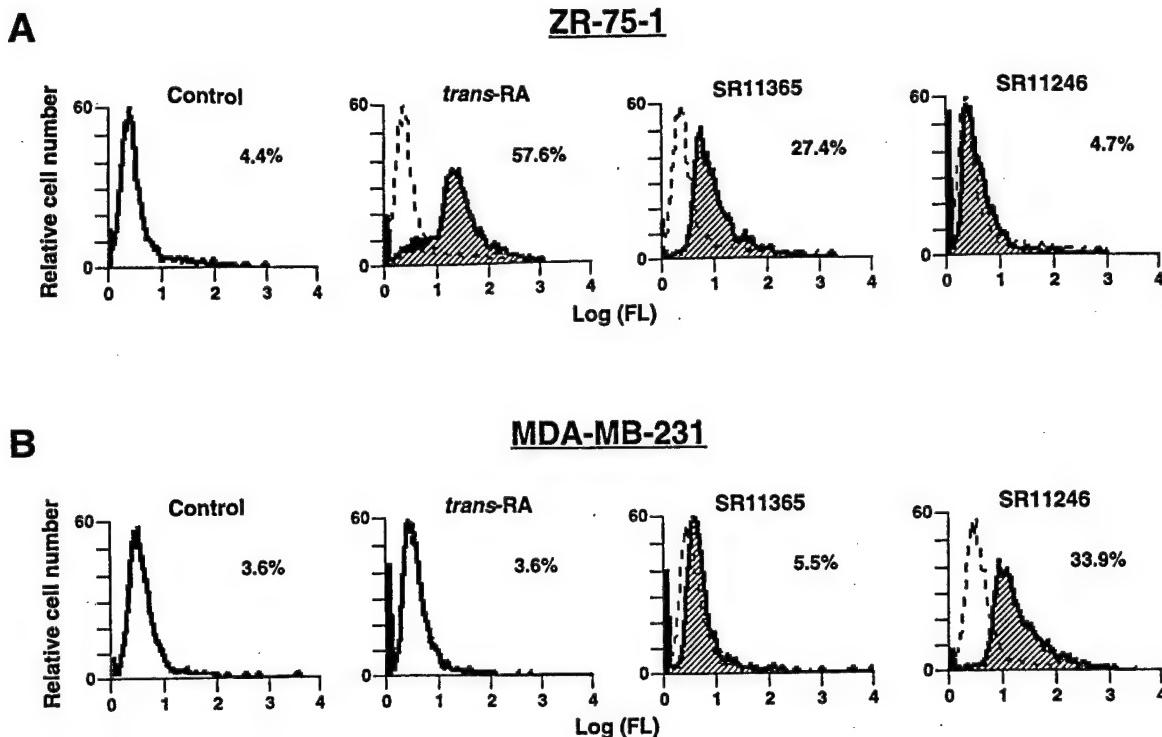


FIG. 2. Induction of apoptosis by retinoids in *trans*-RA-sensitive, estrogen-dependent ZR-75-1 (A) and *trans*-RA-resistant, estrogen-independent MDA-MB-231 (B) breast cancer cells. Breast cancer cells were grown in the presence of the indicated retinoids at  $10^{-6}$  M or *trans*-RA at  $10^{-7}$  M for 48 h. DNA fragmentation was determined by the TdT assay. Representative histograms show relative apoptotic cell number. FL, fluorescence.

the RXR-nur77 heterodimers to the  $\beta$ RARE was comparable to that of the RXR-RAR heterodimers (Fig. 4A). Thus, the unique structure of the  $\beta$ RARE permits binding of both RXR-RAR and RXR/nur77 heterodimers, as previously observed (15, 49). We next carried out transient transfection assays in CV-1 cells, using the RAR $\beta$  promoter linked to the CAT gene (21) as a reporter. As shown in Fig. 4B, cotransfection of RXR $\alpha$  expression vector did not induce reporter transcriptional activity in response to RXR-selective SR11246 or SR11345, a result that suggests that RXR homodimers do not activate the RAR $\beta$  promoter as previously observed (68). Co-

transfection of the nur77 expression vector clearly induced reporter activity in response to these RXR-selective retinoids but not to RAR-selective *trans*-RA or SR11365. When nur77 and RXR expression vectors were cotransfected, a stronger induction of reporter activity was observed when cells were treated with the RXR-selective retinoids but not with *trans*-RA or SR11365. To determine the effect of RAR $\alpha$  on RXR-nur77 activity, we cotransfected the RAR $\alpha$  expression vector together with RXR $\alpha$  and nur77. Addition of RAR $\alpha$  strongly inhibited RXR-selective retinoid-induced reporter activity but significantly enhanced *trans*-RA and SR11365 activity (Fig. 4B). The inhibition of SR11246 and SR11345 activity by RAR $\alpha$  is likely due to competition of RAR $\alpha$  and nur77 for heterodimerization with RXR and binding to the  $\beta$ RARE, which suggests that RXR-selective retinoids SR11246 and SR11345 cannot activate RAR $\beta$  promoter through RXR-RAR heterodimers in CV-1 cells.

**Competitive binding of RXR-RAR and RXR-nur77 heterodimers to the  $\beta$ RARE.** Our observation that RAR $\alpha$  inhibited the transactivation activities of RXR-selective SR11246 and SR11345 on the  $\beta$ RARE (Fig. 4B) suggests that RAR $\alpha$  may compete with nur77 for heterodimerization with RXR and thus prevent nur77 from binding to the  $\beta$ RARE. We therefore carried out gel retardation assay using the  $\beta$ RARE as a probe (Fig. 5). When RAR $\alpha$  protein was added, binding of RXR $\alpha$ -nur77 heterodimers to the  $\beta$ RARE decreased in a RAR $\alpha$  dose-dependent manner. Excess amounts of RAR $\alpha$  permitted binding of RXR $\alpha$ -RAR $\alpha$  heterodimers. Similarly, increasing nur77 protein levels inhibited RXR-RAR heterodimer binding to the  $\beta$ RARE but enhanced RXR-nur77 heterodimer binding. Thus, RAR $\alpha$  and nur77 compete for dimerization with RXR and binding to the  $\beta$ RARE. These data suggest that the

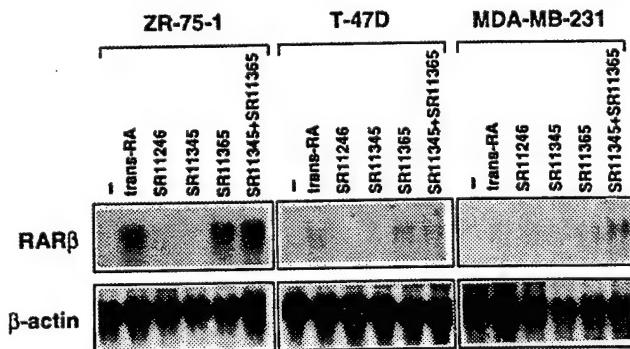


FIG. 3. Effects of RAR-selective SR11365 and RXR-selective SR11246 and SR11345 on RAR $\beta$  gene expression in *trans*-RA-sensitive, estrogen-dependent ZR-75-1 and T-47D and *trans*-RA-resistant, estrogen-independent MDA-MB-231 cells. RNAs were prepared from cells treated with  $10^{-6}$  M RXR-selective SR11246 or SR11345, RAR-selective SR11365, or a combination of SR11345 and SR11365 for 24 h and analyzed for RAR $\beta$  expression by Northern blotting. For comparison, the expression of the  $\beta$ -actin is shown.

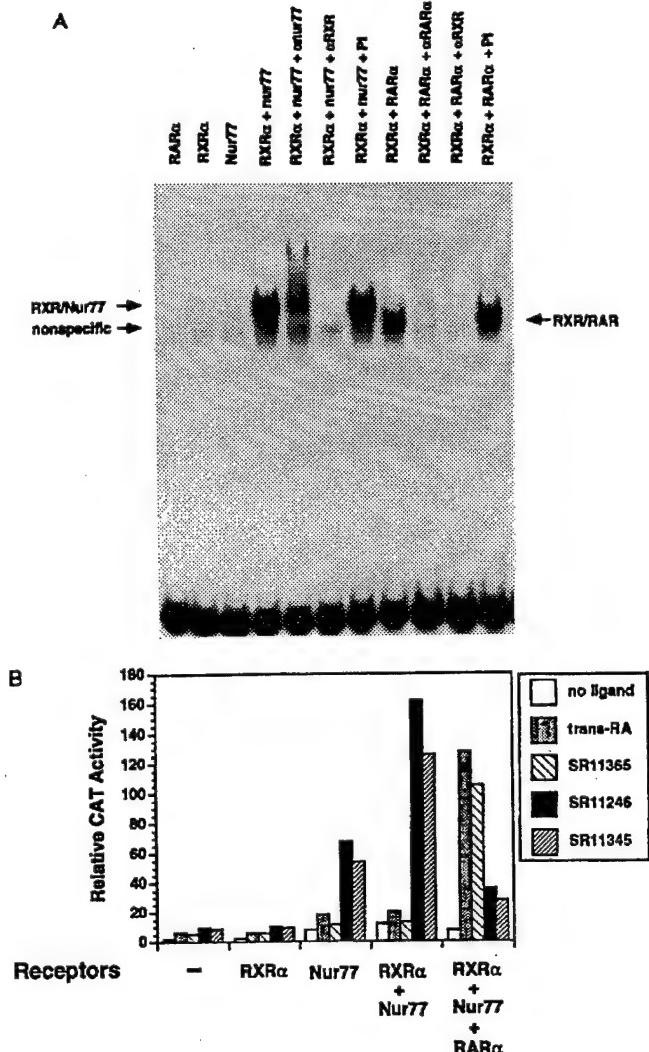


FIG. 4. Binding and transactivation of nur77-RXR $\alpha$  and RAR $\alpha$ -RXR $\alpha$  on the  $\beta$ RARE. (A) Binding of the nur77-RXR $\alpha$  and RXR $\alpha$ -RAR $\alpha$  heterodimers to the  $\beta$ RARE. Equal amounts of in vitro-synthesized nur77 and a N-terminal deleted RXR $\alpha$  (see Materials and Methods) alone or combined were incubated with the  $\beta$ RARE probe at room temperature for 10 min. Mixtures were analyzed by gel retardation. Anti-RXR ( $\alpha$ RXR), anti-RAR $\alpha$  ( $\alpha$ RAR $\alpha$ ), or anti-nur77 ( $\alpha$ nur77) was incubated with receptor proteins for 30 min at room temperature before performance of the assay. For a control, receptor proteins were also incubated with preimmune serum (PI). The  $\beta$ RARE probe sequence was GTAGGGTTCACCGAAAGTTCACT (the NBRE is in boldface). (B) nur77-promoted transactivation of RAR $\beta$  in CV-1 cells. A CAT reporter containing the RAR $\beta$  promoter (19) was transiently transfected into CV-1 cells with the receptor expression vector RXR $\alpha$  (20 ng), nur77 (100 ng), or RAR $\alpha$  (200 ng). After 24 h, cells were treated with the indicated retinoids ( $10^{-6}$  M) or trans-RA ( $10^{-7}$  M) for 24 h, and CAT activities were determined as described elsewhere (65).

relative levels of RAR $\alpha$  and nur77 regulate binding of RXR $\alpha$ -nur77 and RXR $\alpha$ -RAR $\alpha$  to the  $\beta$ RARE. We therefore determined whether different levels of RAR $\alpha$ , RXR $\alpha$ , and nur77 were expressed in *trans*-RA-sensitive ZR-75-1 and in *trans*-RA-resistant MDA-MB-231 cells. Consistent with previous observations (34, 51, 54, 55, 60), RAR $\alpha$  levels were much higher in ZR-75-1 cells than in MDA-MB-231 cells (Fig. 6). However, RXR $\alpha$  and nur77 were equally expressed in both cell lines independently of the presence of *trans*-RA. The high RAR $\alpha$  levels in ZR-75-1 cells suggest that binding of RXR $\alpha$ -RAR $\alpha$  heterodimers to the  $\beta$ RARE may preferentially occur to me-

RXR $\alpha$	2	-	0.5	1	2	4	6	2	2	2	2	2
nur77	-	2	2	2	2	2	2	0.5	1	2	4	6
RXR $\alpha$	+	+	+	+	+	+	+	+	+	+	+	+

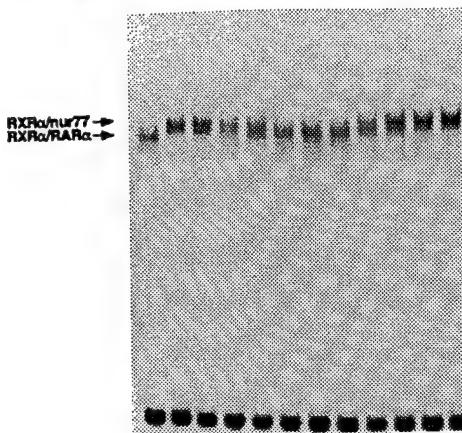


FIG. 5. Competition binding of RXR-RAR and RXR-nur77 heterodimers to the  $\beta$ RARE. To analyze the effect of RAR $\alpha$  on RXR-nur77 heterodimer binding to the  $\beta$ RARE, in vitro-synthesized RAR $\alpha$  protein (1  $\mu$ l) was incubated with in vitro-synthesized nur77 (2  $\mu$ l) in the absence or presence of the indicated amounts (microliters) of in vitro-synthesized RAR $\alpha$  protein and analyzed by gel retardation using the  $\beta$ RARE probe. To analyze the effect of nur77 on RXR $\alpha$ -RAR $\alpha$  heterodimer binding, in vitro-synthesized RXR $\alpha$  protein (1  $\mu$ l) was incubated with in vitro-synthesized RAR $\alpha$  (2  $\mu$ l) in the absence or presence of the indicated amounts ( $\mu$ l) of in vitro-synthesized nur77 and analyzed by gel retardation.

diate the effects of RAR-selective ligands, while the low RAR $\alpha$  levels in MDA-MB-231 cells suggest that RXR-nur77 heterodimer may be predominantly formed with the  $\beta$ RARE to mediate the inhibitory effects of RXR-selective ligands.

Stable expression of RAR $\alpha$  in *trans*-RA-resistant MDA-MB-231 cells favors the RAR pathway over the RXR pathway. Low levels of RAR $\alpha$  in MDA-MB-231 cells should enhance RXR $\alpha$ -nur77 heterodimer formation to mediate the effects of RXR-selective retinoids. To determine whether overexpression of RAR $\alpha$  would allow RXR-RAR heterodimer formation but inhibit that of RXR-nur77, we stably transfected RAR $\alpha$  into this cell line. Two stable clones (MDA-MB-231-RAR $\alpha$ 2 and MDA-MB-231-RAR $\alpha$ 4) that expressed high levels of transfected RAR $\alpha$  (data not shown) were analyzed for their re-

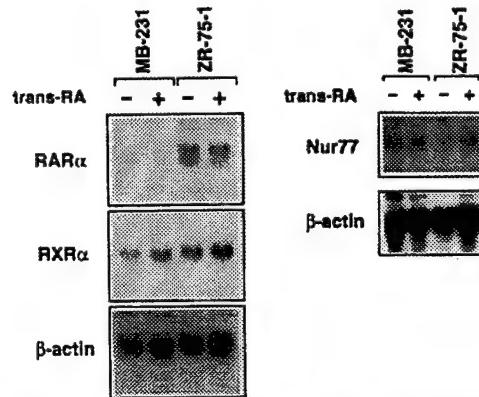


FIG. 6. Expression of RAR $\alpha$ , RXR $\alpha$ , and nur77 in *trans*-RA-sensitive ZR-75-1 and *trans*-RA-resistant MDA-MB-231 cells. Total RNAs, prepared from cells treated with  $10^{-7}$  M *trans*-RA for 24 h, were analyzed for the expression of RAR $\alpha$ , RXR $\alpha$ , and nur77 by Northern blotting. For comparison, the expression of the  $\beta$ -actin is shown.

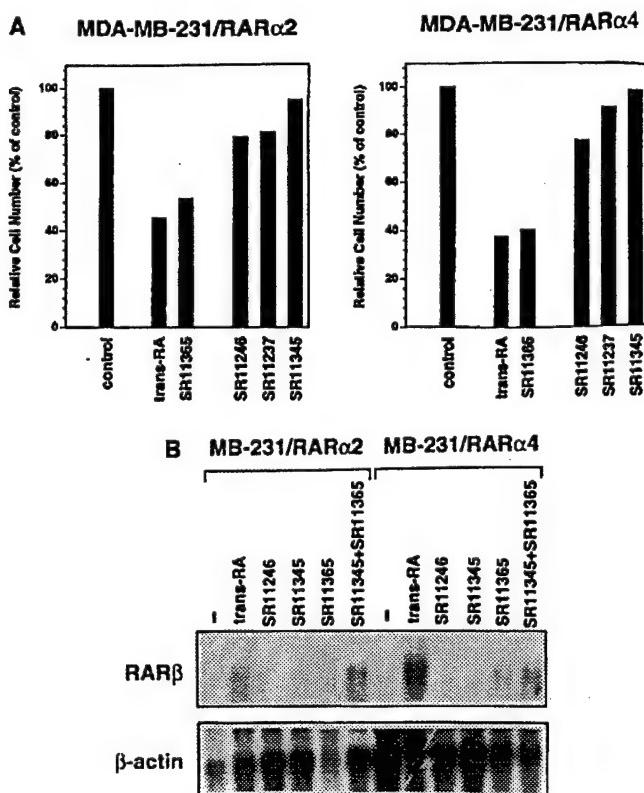


FIG. 7. Effect of stable expression of RAR $\alpha$  on growth inhibition and RAR $\beta$  induction by RXR-selective and RAR-selective retinoids in *trans*-RA-resistant MDA-MB-231 cells. (A) RAR $\alpha$  modulates sensitivity of MDA-MB-231 cells to RAR-selective and RXR-selective retinoids. Stable clones, expressing introduced RAR $\alpha$ , were seeded at 800 cells/well in 96-well plates and treated with the indicated retinoid ( $10^{-6}$  M) or *trans*-RA ( $10^{-7}$  M) for 10 days. The number of viable cells was determined by the MTT assay. (B) Stable expression of RAR $\alpha$  regulates RAR $\beta$  expression in response to RAR-selective and RXR-selective retinoids in MDA-MB-231 cells. Stable MDA-MB-231 clones expressing high levels of RAR $\alpha$  (MB-231/RAR $\alpha$ 2 and MB-231/RAR $\alpha$ 4) were treated with the indicated retinoid ( $10^{-6}$  M) alone or in combination and analyzed for the expression of RAR $\beta$  by Northern blotting. The expression of  $\beta$ -actin was used as the control.

sponses to RAR and RXR class-selective retinoids (Fig. 7A). Compared to their effects in the parental MDA-MB-231 cells (Fig. 1), RAR-selective *trans*-RA at  $10^{-7}$  M and SR11365 at  $10^{-6}$  M were far more potent inhibitors of the stable clones, showing 46 to 62% inhibition, while RXR-selective SR11246, SR11237, and SR11345 were less effective inhibitors, with less than 21% inhibition. We also investigated the effect of RAR $\alpha$  on RAR $\beta$  expression in MDA-MB-231 cells by Northern blotting (Fig. 7B). In contrast to their effects on the parental cells (Fig. 3), RAR-selective *trans*-RA and SR11365 strongly induced RAR $\beta$  expression in both clones, while RXR-selective retinoids SR11246 and SR11345 did not. These results demonstrate that low RAR $\alpha$  expression in MDA-MB-231 cells is responsible for the increased ability of RXR-selective retinoids and the decreased ability of RAR-selective retinoids to induce RAR $\beta$  expression and growth inhibition. The fact that the extent of growth inhibition and RAR $\beta$  expression level by these receptor class-selective retinoids in the stable clones were similar to those in *trans*-RA-sensitive ZR-75-1 and T-47D cells (Fig. 1 and 3) suggests that the differential effects of retinoids on certain *trans*-RA-sensitive and -resistant breast cancer cell lines depend on different levels of RAR $\alpha$  expression.

**Binding of nuclear proteins from ZR-75-1 and MDA-MB-231 cells to the  $\beta$ RARE.** To provide direct evidence that relative levels of RXR, RAR $\alpha$ , and nur77 in *trans*-RA-sensitive ZR-75-1 and MDA-MB-231 cells allowed different complex formation on the  $\beta$ RARE, we prepared nuclear proteins from ZR-75-1 and MDA-MB-231 cells and analyzed their binding to the  $\beta$ RARE (Fig. 8). Nuclear proteins from ZR-75-1 cells formed several strong complexes with the  $\beta$ RARE. When they were incubated with anti-RXR antibody, the slowly migrating complexes were inhibited. When anti-RAR $\alpha$  antibody was

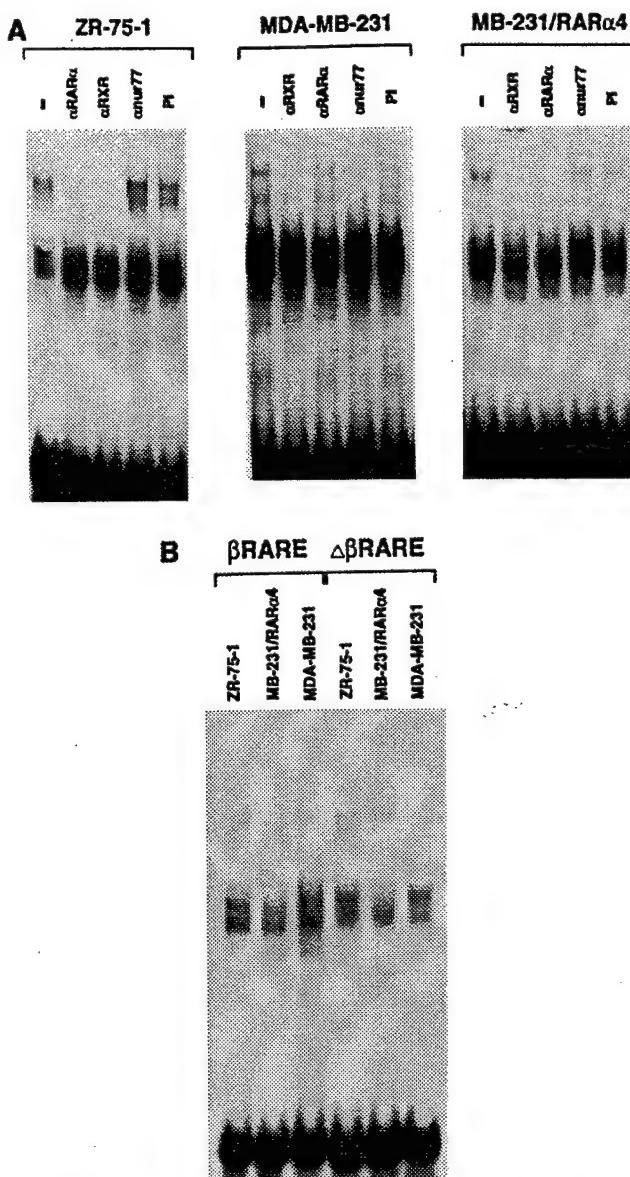


FIG. 8.  $\beta$ RARE binding activities of nuclear proteins from ZR-75-1, MDA-MB-231, and MDA-MB-231-RAR $\alpha$ 4 cells. (A) Nuclear proteins from ZR-75-1 (2  $\mu$ g), MDA-MB-231 (5  $\mu$ g), and MDA-MB-231-RAR $\alpha$ 4 (2  $\mu$ g) cells were analyzed by gel retardation assay using  $\beta$ RARE as a probe. When antibody (designated as in Fig. 4A) was used, it (1  $\mu$ l) was incubated with nuclear proteins for 30 min at room temperature before performance of the assay. Preimmune serum (PI) was used for control. (B) Comparison of  $\beta$ RARE and  $\Delta\beta$ RARE binding of nuclear proteins from ZR-75-1 (2  $\mu$ g), MDA-MB-231 (2  $\mu$ g), and MDA-MB-231-RAR $\alpha$ 4 (2  $\mu$ g) cells by gel retardation assay. The  $\Delta\beta$ RARE probe sequence was GTAGGGTTCACCGTGAGTTCAAGTC (mutated nucleotides compared to  $\beta$ RARE are indicated in boldface).

used, some of the slowly migrating complexes were also abolished. However, anti-nur77 antibody did not show any detectable effect on the binding. These data demonstrate that RXR and RAR $\alpha$  are mainly responsible for  $\beta$ RARE binding in ZR-75-1 cells. When nuclear proteins from MDA-MB-231 cells were analyzed, we observed weak  $\beta$ RARE binding complexes, which could be inhibited by anti-RXR and anti-nur77 antibodies but not by anti-RAR $\alpha$  antibody. Thus, expression of RXR and nur77 in MDA-MB-231 cells (Fig. 6) could contribute to the  $\beta$ RARE binding activities. To determine whether overexpression of RAR $\alpha$  in MDA-MB-231 cells could prevent RXR-nur77 binding as we observed by using *in vitro*-synthesized receptor proteins (Fig. 5), we analyzed the binding of nuclear proteins prepared from MDA-MB-231-RAR $\alpha$ 4 cells. As shown in Fig. 8A, the nuclear proteins formed a strong complex with the  $\beta$ RARE, which could be completely inhibited by either anti-RXR antibody or anti-RAR $\alpha$  antibody but not by anti-nur77 antibody, indicating that overexpression of RAR $\alpha$  in MDA-MB-231 cells inhibited RXR-nur77 heterodimer binding and permitted RXR-RAR heterodimer binding. To further determine the nature of the binding complexes from different cell lines, we used a mutated  $\beta$ RARE ( $\Delta\beta$ RARE), in which two adenine nucleotides in the spacing region of the  $\beta$ RARE were mutated. The mutations do not affect binding of RXR-RAR heterodimers but abolish RXR-nur77 binding (66) and thereby allow distinction of RXR-RAR heterodimers from RXR-nur77 heterodimers. When nuclear proteins from ZR-75-1, MDA-MB-231, and MDA-MB-231-RAR $\alpha$ 4 were analyzed on the  $\Delta\beta$ RARE, we observed a strong binding of nuclear proteins from ZR-75-1 and MDA-MB-231-RAR $\alpha$ 4 cells, similar to that observed with the  $\beta$ RARE. In contrast, nuclear proteins from MDA-MB-231 cells did not show any detectable binding on the  $\Delta\beta$ RARE, demonstrating that the binding complex that we observed on the  $\beta$ RARE might represent RXR-nur77 heterodimer binding.

## DISCUSSION

**Breast cancer cell growth inhibition and apoptosis induction by RXR-selective retinoids.** Although conventional retinoids show promise in animal models as preventive agents against breast cancer, their anticancer effects appear to be limited to *trans*-RA-sensitive tumors, whereas the more aggressive, estrogen-independent tumors are usually refractory (14, 34, 54, 55, 60). In this study, we demonstrated that several RXR-selective retinoids inhibit the growth and induce the apoptosis of *trans*-RA-resistant MDA-MB-231 cells (Fig. 1), provided that they are also capable of activating the RARs or are used in combination with RAR-selective retinoids. Efficient growth inhibition by RXR-selective retinoids appears to be cell type specific because it was observed in *trans*-RA-resistant MDA-MB-231 cells but not in *trans*-RA-sensitive ZR-75-1 cells (Fig. 1). In ZR-75-1 cells, RAR-selective SR11365 was much more effective than RXR-selective SR11246 in inhibiting the growth and inducing apoptosis (Fig. 1 and 2). Thus, different retinoid signaling pathways preferentially operate in *trans*-RA-sensitive and *trans*-RA-resistant breast cancer cells to mediate retinoid-induced growth inhibition.

Although the RXR pathway is clearly involved in *trans*-RA-resistant MDA-MB-231 breast cancer cell growth inhibition, a clear growth inhibition by RXR-selective retinoids required a longer period of treatment (Fig. 1) than that by RAR-selective retinoids, which usually inhibit the growth of *trans*-RA-sensitive breast cancer cells over a period of 3 to 4 days of treatment (data not shown). This observation suggests that the effects of RXR-selective retinoids may involve a mechanism different

from that utilized by RAR-selective retinoids. We showed that activation of RXR alone was insufficient for growth inhibition and that activation of RAR appeared to be required, as indicated by our observation that RXR-selective SR11237, SR11246, and SR11247, which at 1  $\mu$ M have the ability to slightly activate the RAR $\beta$  (Table 1), can significantly inhibit the growth of MDA-MB-231 cells (Fig. 1), whereas the far more RXR-selective SR11345, which activates RXR $\alpha$  comparably to the other RXR-selective retinoids (Table 1), did not significantly inhibit MDA-MB-231 cell growth (Fig. 1). Furthermore, RXR-selective SR11345 and RAR-selective SR11365, which alone were ineffective inhibitors, or combination strongly prevented MDA-MB-231 cell growth (Fig. 1). The biological activities of RXR-selective retinoids have been described in several studies (6, 17, 47). Activation of RXR was reported as essential for inducing apoptosis in HL-60 leukemia cells (47). RXR-selective retinoid LGD1069 effectively inhibited the tumor development in the *N*-nitroso-*N*-methylurea-induced rat mammary tumor model (17). Because of their increased efficacy against malignant, *trans*-RA-resistant, estrogen-independent breast cancer growth, RXR-selective retinoids may be useful for chemoprevention and chemotherapy of breast cancer.

How RXR-selective retinoids inhibit the growth and induce the apoptosis of *trans*-RA-resistant MDA-MB-231 and other cancer cells remains to be fully elucidated. Induction of growth inhibition and apoptosis of breast cancer cells by retinoids may involve different retinoid receptors and different mechanisms, depending on types of retinoids and cell lines (11, 12, 33, 34, 53–55, 60). The effects of *trans*-RA may be mediated by RAR $\alpha$  and RAR $\beta$  (33, 34, 53–55, 60), whereas activation of RAR $\gamma$  may be required for other retinoids, such as 4-HPR (11, 12). Our data presented here suggest that induction of RAR $\beta$  may be involved. This is consistent with previous observations that RAR $\beta$  could mediate the growth-inhibitory effect of *trans*-RA in breast cancer cells (33, 34, 53). RAR $\beta$  was induced by *trans*-RA only in *trans*-RA-sensitive ZR-75-1 and T-47D, not in *trans*-RA-resistant MDA-MB-231, breast cancer cells (34). In addition, introduction of RAR $\beta$  into RAR $\beta$ -negative MDA-MB-231 breast cancer cell lines restored *trans*-RA-induced growth inhibition (33, 34, 53), while inhibition of RAR $\beta$  activity in the RAR $\beta$ -positive ZR-75-1 cells with an antisense construct abolished growth inhibition by *trans*-RA (34). Furthermore, enhancement of RAR $\beta$  levels has been found to correlate with senescence in normal mammary epithelial cells (58). In *trans*-RA-sensitive ZR-75-1 and T-47D cells, RAR $\beta$  expression was strongly induced by RAR-selective *trans*-RA and SR11365, which also inhibited growth and induced apoptosis, whereas RAR $\beta$  expression was not induced by RXR-selective SR11246 and SR11345, which only poorly inhibited growth and only weakly induced apoptosis (Fig. 3). The fact that RXR-selective retinoids could induce RAR $\beta$  expression in *trans*-RA-resistant MDA-MB-231 cells (Fig. 3) suggests that induction of RAR $\beta$  may contribute to their effects on MDA-MB-231 cells. However, because *trans*-RA and SR11365 induced RAR $\beta$  to levels similar to those induced by RXR-selective retinoids SR11246 and SR11345 in MDA-MB-231 cells (Fig. 3) but were poor growth inhibitors (Fig. 1), mechanisms other than RAR $\beta$  induction may also be involved.

SR11345 synergized with RAR-selective retinoids to inhibit MDA-MB-231 cell growth (Fig. 1). Such synergism of RAR- and RXR-selective retinoids has recently been observed in the activation of several RA-responsive genes, including RAR $\beta$ , during embryonal carcinoma cell differentiation (52) and in NB4 acute promyelocytic leukemia cells (4). The synergism that we observed here may in part arise from induction and

activation of RAR $\beta$ . Growth inhibition of MDA-MB-231 cells may require both induction and activation of RAR $\beta$  because RXR-specific SR11345 is a much less effective inhibitor than other RXR-selective retinoids (Fig. 1), which also slightly activate RAR $\beta$  (Table 1). Enhanced induction of RAR $\beta$  in MDA-MB-231 cells by the combination of RXR-specific SR11345 and RAR-selective SR11365 (Fig. 3) may also contribute to their synergistic growth inhibition.

**Regulation of RAR $\beta$  expression.** The  $\beta$ RARE in the RAR $\beta$  promoter is responsible for regulating RAR $\beta$  expression by retinoids (10, 21, 57). Our observation in gel shift assays that efficient binding to the  $\beta$ RARE occurred by heterodimerization of RXR $\alpha$  with nur77 or RAR $\alpha$  but not by either receptor alone (Fig. 4A) confirms that the  $\beta$ RARE binds both RXR-RAR and RXR-nur77 heterodimers (15, 49). Activation of the  $\beta$ RARE by *trans*-RA is caused by binding and activation of RAR-RXR heterodimers, in which RXR functions as a silent partner (15, 30, 38), while activation of the  $\beta$ RARE by RXR-selective retinoids occurs on binding of RXR-nur77 heterodimers (15, 49). Cotransfection of RXR $\alpha$  and nur77 strongly activated RAR $\beta$  promoter activity in response to RXR-selective SR11246 or SR11345 but not to RAR-selective *trans*-RA or SR11365 (Fig. 4B). Thus, the  $\beta$ RARE can be activated by either a RAR-selective or RXR-selective retinoid signaling pathway through binding of RXR-RAR or RXR-nur77, respectively. This is reminiscent of a previous observation that the  $\beta$ RARE bound strongly to a RXR-containing complex other than RXR-RAR in S91 melanoma cell extracts (56). Because nur77 expression is induced by several growth factors having different signal transduction pathways (20, 39), the binding and activation of the  $\beta$ RARE by nur77 and retinoid receptors will mediate the convergence of retinoid and growth factor signaling pathways.

Activation of the  $\beta$ RARE by the RAR pathway or RXR pathway depends on the breast cancer cell type. In *trans*-RA-sensitive cell lines such as ZR-75-1 and T-47D, the expression of RAR $\beta$  was highly induced by RAR-selective retinoids but not by RXR-selective retinoids, whereas in *trans*-RA-resistant MDA-MB-231 cells, RAR $\beta$  expression was induced by RXR-selective retinoids (Fig. 3). Because both RXR $\alpha$  and nur77 are well expressed in MDA-MB-231 cells (Fig. 6), induction of RAR $\beta$  by RXR-selective retinoids is likely mediated by activation of RXR-nur77 heterodimers on the  $\beta$ RARE. This is further supported by our observation that binding of nuclear proteins prepared from MDA-MB-231 cells to the  $\beta$ RARE may represent RXR-nur77 heterodimers (Fig. 8). Such cell-type-specific activation of the  $\beta$ RARE has been observed previously. In CV-1 cells, RXR-selective retinoids did not appreciably transactivate the DR-5  $\beta$ RARE even in the presence of transfected RAR and RXR (15, 30). However, in P19 or F9 embryonal carcinoma cells, RXR ligands contributed to transcriptional activation of genes containing DR-5 elements (4, 40, 52). The cell-type-specific activation of the  $\beta$ RARE is likely due to the relative levels of nuclear receptors that bind the  $\beta$ RARE and modulate its activity.

Although RXR-selective retinoids could induce RAR $\beta$  expression in MDA-MB-231 cells, we observed a stronger induction of RAR $\beta$  when cells were treated with combination of RAR-selective and RXR-selective retinoids (Fig. 3). The strong induction of RAR $\beta$  by the combination may be due to additive effect of RXR-RAR and RXR-nur77 heterodimers, since RAR-selective retinoids by themselves could also slightly induce RAR $\beta$  probably due to low levels of RAR $\alpha$  expressed in these cells. Recently, it was reported that binding of RAR-selective retinoids to RXR-RAR heterodimers allowed binding and activation of RXR-RAR heterodimers by RXR-selective

retinoids (4, 40). Thus, it is also possible that the strong induction of RAR $\beta$  that we observed by the combination of RAR-selective and RXR-selective retinoids is due to activation of RXR-RAR heterodimers prebound with RAR-selective retinoid.

**RAR $\alpha$  regulates both RAR and RXR pathways.** *trans*-RA-sensitive and -resistant breast cancer cell lines display different responses to retinoid receptor class-selective ligands. RAR-selective retinoids are potent RAR $\beta$  inducers and growth inhibitors in *trans*-RA-sensitive ZR-75-1 cells, while RXR-selective retinoids effectively induce RAR $\beta$  and inhibit the growth of *trans*-RA-resistant MDA-MB-231 cells (Fig. 1 and 3). The observation that the  $\beta$ RARE can be activated by either RXR-RAR or RXR-nur77 suggests that the pathway that mediates growth inhibition and RAR $\beta$  induction may largely depend on the relative levels of RAR $\alpha$ , RXR $\alpha$ , and nur77. In lung cancer cell lines, nur77 expression is associated with *trans*-RA resistance (66) and could be critical in regulating RAR and RXR activities. However, in breast cancer cell lines, ZR-75-1 and MDA-MB-231 cell lines express similar levels of RXR $\alpha$  and nur77, while RAR $\alpha$  varies, being highly expressed in the former and underexpressed in the latter (Fig. 6), as has been previously observed (34, 51, 55, 60). Thus, RAR $\alpha$  levels appear to be most important for determining whether the RAR or RXR pathway will regulate growth inhibition by retinoids. High RAR $\alpha$  levels in *trans*-RA-sensitive ZR-75-1 cells may permit formation of RXR-RAR heterodimers that bind to the  $\beta$ RARE (Fig. 5 and 8) to mediate the effects of RAR-selective retinoids in inducing RAR $\beta$  expression and growth inhibition (34) but prevent RXR $\alpha$  from forming RXR-nur77 heterodimers (Fig. 5 and 8) so that RXR-selective retinoids are unable to inhibit growth or induce apoptosis despite the abundant expression of nur77. In contrast, low RAR $\alpha$  levels in *trans*-RA-insensitive MDA-MB-231 cells (Fig. 6) allow formation of RXR-nur77 heterodimers (Fig. 8) that bind to the  $\beta$ RARE to mediate RAR $\beta$  expression and may be responsible for growth inhibition in the presence of RXR-selective retinoids. The importance of RAR $\alpha$  levels in determining the regulatory pathway is supported by our gel retardation (Fig. 5 and 8) and transfection assay results (Fig. 4B). Gel retardation indicates that binding of RXR-RAR or RXR-nur77 heterodimers to the  $\beta$ RARE largely depends on RAR $\alpha$  protein levels (Fig. 5 and 8). Overexpression of RAR $\alpha$  in MDA-MB-231 cells allowed binding of RXR-RAR heterodimers and prevented binding of RXR-nur77 heterodimers to the  $\beta$ RARE (Fig. 5 and 8). In transient transfection assays, cotransfection of RAR $\alpha$  inhibited RXR-selective retinoid-induced RXR-nur77 heterodimer activity on the RAR $\beta$  promoter (Fig. 4B). Furthermore, stable expression of RAR $\alpha$  in MDA-MB-231 cells strongly enhanced growth inhibition (Fig. 7A) and RAR $\beta$  induction (Fig. 7B) by RAR-selective retinoids and decreased the inhibitory effects of RXR-selective retinoids. Thus, high RAR $\alpha$  levels favor formation of RAR-RXR heterodimers and the RAR signaling pathway in breast cancer cells, while low RAR $\alpha$  levels favor the formation of nur77-RXR heterodimers and the RXR signaling pathway. This retinoid signaling switch may play an important role in regulating breast cancer cell growth in response to different growth factor and retinoid stimuli.

In summary, we have demonstrated that RXR-selective retinoids inhibit the growth and induce the apoptosis of *trans*-RA-resistant MDA-MB-231 breast cancer cells, which appears to be mediated through RXR-nur77 heterodimers that bind and activate the  $\beta$ RARE in the presence of RXR-selective retinoids, resulting in induction of RAR $\beta$ , which may then be activated by RAR-selective retinoids to initiate secondary bio-

logical responses. RXR-nur77 heterodimer formation in *trans*-RA-resistant MDA-MB-231 cells is favored by very low RAR $\alpha$  levels, whereas high expression of RAR $\alpha$  in *trans*-RA-sensitive ZR-75-1 cells favors formation of RXR-RAR heterodimers that bind and activate the  $\beta$ RARE in response to RAR-selective ligands. Thus, the convergence and switch of RAR-dependent and RXR-dependent signaling on the  $\beta$ RARE is very likely regulated by relative RAR $\alpha$  levels. Our findings that a RXR signaling pathway can mediate growth inhibition and apoptosis induction and the additive to synergistic effects of a RAR-selective and RXR-selective retinoid combination on *trans*-RA-resistant MDA-MB-231 cell growth may provide a therapeutic opportunity to inhibit the growth of more invasive, *trans*-RA-resistant breast cancer by using lower retinoid doses to reduce toxicity.

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#### REFERENCES

- Bartsch, D., B. Boye, C. Baust, H. zur Hausen, and E. Schwarz. 1992. Retinoic acid-mediated repression of human papillomavirus 18 transcription and different ligand regulation of the retinoic acid receptor  $\beta$  gene in non-tumorigenic and tumorigenic HeLa hybrid cells. *EMBO J.* 11:2283-2291.
- Boccard, F., L. Canobbio, M. Resasco, A. U. Decensi, G. Pastorino, and F. Bremia. 1990. Phase II study of tamoxifen and high-dose retinyl acetate in patients with advanced breast cancer. *J. Cancer Res. Clin. Oncol.* 116:503-506.
- Cassidy, J., M. Lippman, A. Lacroix, and G. Peck. 1982. Phase II trial of 13-cis-retinoic acid in metastatic breast cancer. *Eur. J. Cancer Clin. Oncol.* 18:925-928.
- Chen, J.-Y., J. Clifford, C. Zusi, J. Starrett, D. Tortolani, J. Ostrowski, P. R. Reczel, P. Chambon, and H. Gronemeyer. 1996. Two distinct actions of retinoid-receptor ligands. *Nature* 382:819-822.
- Clifford, J. L., M. Petkovich, P. Chambon, and R. Lotan. 1990. Modulation by retinoids of mRNA levels for nuclear retinoic acid receptors in murine melanoma cells. *Mol. Endocrinol.* 4:1546-1555.
- Davis, K. D., T. J. Berrodin, J. E. Stelmach, J. D. Winkler, and M. A. Lazar. 1994. Endogenous retinoid X receptors can function as hormone receptors in pituitary cells. *Mol. Cell. Biol.* 14:7105-7110.
- Dawson, M. I., W. R. Chao, P. Ping, L. Jong, P. D. Hobbs, C. Rudd, T. C. Quick, R. M. Niles, X.-K. Zhang, A. Lombardo, K. R. Ely, B. Shroot, and J. A. Fontana. 1995. Correlation of retinoid binding affinity to RAR $\alpha$  with retinoid inhibition of growth of estrogen receptor-positive MCF-7 mammary carcinoma cells. *Cancer Res.* 55:4446-4451.
- Dawson, M. I., L. Jong, P. D. Hobbs, J. F. Cameron, W. R. Chao, M. Pfahl, M. O. Lee, and B. Shroot. 1995. Conformational effects on retinoid receptor selectivity. 2. Effects of retinoid bridging group on retinoid X receptor activity and selectivity. *J. Med. Chem.* 38:3368-3383.
- Dejean, A., L. Bougueret, K.-H. Grzeschik, and P. Tiollais. 1986. Hepatitis B virus DNA integration in a sequence homologous to *v-erb-A* and steroid receptor genes in a hepatocellular carcinoma. *Nature* 322:70-72.
- de The, H., M. del Mar Vivanco-Ruiz, P. Tiollais, H. Stunnenberg, and A. Dejean. 1990. Identification of a retinoic acid responsive element in the retinoic acid receptor  $\beta$  gene. *Nature* 343:177-180.
- Fanjul, A. N., D. Delia, M. A. Pierotti, D. Rideout, J. Qiu, and M. Pfahl. 1996. 4-Hydroxyphenyl retinamide is a highly selective activator of retinoid receptors. *J. Biol. Chem.* 271:22441-22446.
- Fanjul, A. N., H. Bouterfa, M. Dawson, and M. Pfahl. 1996. Potential role for retinoic acid receptor- $\gamma$  in the inhibition of breast cancer cells by selective retinoids and interferons. *Cancer Res.* 56:1571-1577.
- Fontana, J. A. 1987. Interaction of retinoids and tamoxifen on the inhibition of human mammary carcinoma cell proliferation. *Exp. Cell. Biol.* 55:136-144.
- Fontana, J. A., P. D. Hobbs, and M. I. Dawson. 1988. Inhibition of mammary carcinoma growth by retinoidal benzoic acid derivatives. *Exp. Cell. Biol.* 56:254-263.
- Forman, B. M., K. Umesono, J. Chen, and R. M. Evans. 1995. Unique response pathways are established by allosteric interactions among receptors. *Cell* 81:541-550.
- Gebert, J. F., N. Moghal, J. V. Frangioni, D. J. Sugabaker, and B. G. Neel. 1991. High frequency of retinoic acid receptor  $\beta$  abnormalities in human lung cancer. *Oncogene* 6:1859-1868.
- Gottardis, M. M., E. D. Bischoff, M. A. Shirley, M. A. Wagoner, W. W. Lamph, and R. A. Heyman. 1996. Chemoprevention of mammary carcinoma by LGD1069 (Targretin): an RXR-selective ligand. *Cancer Res.* 56:5566-5570.
- Grubbs, C. J., R. C. Moon, M. B. Sporn, and D. L. Newton. 1977. Inhibition of mammary cancer by retinyl methyl ether. *Cancer Res.* 37:599-602.
- Gudas, L. J., M. B. Sporn, and A. B. Roberts. 1994. Cellular biology and biochemistry of the retinoids, p. 443-520. In M. B. Sporn, A. B. Roberts, and D. S. Goodman (ed.), *The retinoids*. Raven Press, New York, N.Y.
- Hazel, T. G., D. Nathans, and L. F. Lau. 1988. A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. *Proc. Natl. Acad. Sci. USA* 85:8444-8448.
- Hoffmann, B., J. M. Lehmann, X.-K. Zhang, T. Hermann, G. Graupner, and M. Pfahl. 1990. A retinoic acid receptor-specific element controls the retinoic acid receptor- $\beta$  promoter. *Mol. Endocrinol.* 4:1734-1743.
- Houle, B., F. Ledue, and W. E. C. Bradley. 1991. Implication of RAR $\beta$  in epidermoid (squamous) carcinoma cells. *Exp. Cell Res.* 195:163-170.
- Houle, B., C. Rochette-Egly, and W. E. C. Bradley. 1993. Tumor-suppressive effect of the retinoic acid receptor  $\beta$  in human epidermoid lung cancer cells. *Proc. Natl. Acad. Sci. USA* 90:985-989.
- Hu, L., D. L. Crowe, J. G. Rheinwald, P. Chambon, and L. Gudas. 1991. Abnormal expression of retinoic acid receptors and keratin 19 by human oral and epidermal squamous cell carcinoma cell lines. *Cancer Res.* 51:3972-3981.
- Kagechika, H., E. Kawachi, Y. Hashimoto, T. Himi, and K. Shudo. 1988. Retinobenzoic acids. 1. Structure-activity relationships of aromatic amides with retinoidal activity. *J. Med. Chem.* 31:2182-2192.
- Kagechika, H., E. Kawachi, G. Saito, H. Fukusawa, Y. Hashimoto, and K. Shudo. 1997. Comparison of differentiation between NB4 and HL-60 cells induced by retinoids, abstr. B-27. In *Abstracts of Mechanisms of action of retinoids, vitamin D, and steroid hormones AACR Special Conference*.
- Kamel, Y., L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S.-C. Lin, R. A. Heyman, D. W. Rose, C. K. Glass, and M. G. Rosenfeld. 1996. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* 85:403-414.
- Kastner, P., M. Mark, and P. Chambon. 1995. Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell* 83:859-869.
- Koga, M., and R. L. Sutherland. 1991. Retinoic acid acts synergistically with 1,25-dihydroxyvitamin D $_3$  or antiestrogen to inhibit T-47D human breast cancer cell proliferation. *J. Steroid Biochem. Mol. Biol.* 39:455-460.
- Kurokawa, R., J. DiRenzo, M. Boehm, J. Sugarman, B. Gloss, M. G. Rosenfeld, R. A. Heyman, and C. K. Glass. 1994. Regulation of retinoid signalling by receptor polarity and allosteric binding. *Nature* 371:528-531.
- Lala, D. S., R. Mukherjee, I. G. Schulman, S. S. Canan Koch, L. J. Darashahi, A. M. Nadzan, G. E. Croston, R. M. Evans, and R. A. Heyman. 1996. Activation of specific RXR heterodimers by an antagonist of RXR homodimers. *Nature* 383:450-453.
- Lee, M.-O., Y. Liu, and X.-K. Zhang. 1995. A retinoic acid response element that overlaps an estrogen response element mediates multihormonal sensitivity in transcriptional activation of the lactoferrin gene. *Mol. Cell Biol.* 15:4194-4207.
- Li, X.-S., Z.-M. Shao, M. H. Shekh, J. L. Elseman, D. Sentz, A. M. Jetten, J.-H. Chen, M. I. Dawson, S. Alsner, A. K. Rishi, P. Gutierrez, L. Schnapper, and J. A. Fontana. 1995. Retinoic acid nuclear receptor  $\beta$  inhibits breast carcinoma anchorage independent growth. *J. Cell. Physiol.* 165:449-458.
- Liu, Y., M.-O. Lee, H.-G. Wang, Y. Li, Y. Hashimoto, M. Klaus, J. C. Reed, and X.-K. Zhang. 1996. RAR $\beta$  mediates the growth inhibitory effect of retinoic acid by inducing cell apoptosis in human breast cancer cells. *Mol. Cell. Biol.* 16:1138-1149.
- Liu, Z.-C., S. W. Smith, K. A. McLaughlin, L. M. Schwartz, and B. A. Osborne. 1994. Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene nur77. *Nature* 367:281-284.
- Lotan, R. 1981. Effect of vitamin A and its analogs (retinoids) on normal and neoplastic cells. *Biochim. Biophys. Acta* 605:33-91.
- Lotan, R., X. C. Xu, S. M. Lippman, J. Y. Ro, J. S. Lee, J. J. Lee, and W. K. Hong. 1995. Suppression of retinoic acid receptor- $\beta$  in premalignant oral lesions and its upregulation by isotretinoin. *N. Engl. J. Med.* 332:1405-1410.
- Mangelsdorf, D. J., and R. M. Evans. 1995. The RXR heterodimers and orphan receptors. *Cell* 83:841-850.
- Milbrandt, J. 1988. Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. *Neuron* 1:183-188.
- Minucci, S., M. Leid, R. Toyama, J.-P. Saint-Jeannet, V. J. Peterson, V. Horn, J. E. Ishmael, N. Bhattacharya, A. Dey, L. B. Dawid, and K. Ozato. 1997. Retinoid X receptor (RXR) within the RXR-retinoic acid receptor heterodimer binds its ligand and enhances retinoid-dependent gene expression. *Mol. Cell. Biol.* 17:644-655.
- Miyaura, N., and A. Suzuki. 1995. Palladium-catalyzed cross-coupling reactions of organoboron compounds. *Chem. Rev.* 95:2457-2483.

42. Modiano, M., W. Dalton, S. Lippman, L. Joffe, A. R. Booth, and F. L. Meyskens, Jr. 1990. Phase II study of fenretinide (N-[4-hydroxyphenyl]retinamide) in advanced breast cancer and melanoma. *Invest. New Drugs* 8:317-319.
43. Moon, R. C., and R. G. Mehta. 1990. Chemoprevention of mammary cancer by retinoids. *Basic Life Sci.* 52:213-224.
44. Moon, R. C., J. F. Pritchard, R. G. Mehta, C. T. Monides, C. F. Thomas, and N. M. Dinger. 1989. Suppression of rat mammary cancer development by N-(4-hydroxyphenyl)retinamide (4-HPR) following surgical removal of first palpable tumor. *Carcinogenesis* 10:1645-1649.
45. Moon, R. C., H. J. Thompson, P. J. Becci, C. J. Grubbs, R. J. Gander, D. L. Newton, J. M. Smith, S. L. Phillips, W. R. Henderson, L. T. Mullen, C. C. Brown, and M. B. Sporn. 1979. N-(4-Hydroxyphenyl)retinamide, a new retinoid for prevention of breast cancer in the rat. *Cancer Res.* 39:1339-1346.
46. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65:55-63.
47. Nagy, L., V. A. Thomazy, G. L. Shipley, L. Fesus, W. Lamph, R. A. Heyman, R. A. Chandraratna, and P. J. Davies. 1995. Activation of retinoid X receptors induces apoptosis in HL-60 cell lines. *Mol. Cell. Biol.* 15:3540-3551.
48. Nervi, C., T. M. Volleberg, M. D. George, A. Zelenit, P. Chambon, and A. M. Jetten. 1991. Expression of nuclear retinoic acid receptors in normal tracheobronchial cells and in lung carcinoma cells. *Exp. Cell Res.* 195:163-170.
49. Perlmann, T., and L. Jansson. 1995. A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and Nur77. *Genes Dev.* 9:769-782.
50. Roberts, A. B., and M. B. Sporn. 1984. Cellular biology and biochemistry of the retinoids, p. 209-286. In M. B. Sporn, A. B. Roberts, and D. S. Goodman (ed.), *The retinoids*. Academic Press, Orlando, Fla.
51. Roman, S. D., C. L. Clarke, R. E. Hall, I. E. Alexander, and R. L. Sutherland. 1992. Expression and regulation of retinoic acid receptors in human breast cancer cells. *Cancer Res.* 52:2236-2242.
52. Roy, B., R. Taneja, and P. Chambon. 1995. Synergistic activation of retinoic acid (RA)-responsive genes and induction of embryonal carcinoma cell differentiation by an RA receptor  $\alpha$  (RAR $\alpha$ ), RAR $\beta$ , or RAR $\gamma$ -selective ligand in combination with a retinoid X receptor-specific ligand. *Mol. Cell. Biol.* 15:6481-6487.
53. Seewaldt, V. L., B. S. Johnson, M. B. Parker, S. J. Collins, and K. Swisshein. 1995. Expression of retinoic acid receptor  $\beta$  mediates retinoic acid-induced growth arrest and apoptosis in breast cancer cells. *Cell Growth Differ.* 6:1077-1088.
54. Sheikh, M. S., Z. M. Shao, J. C. Chen, A. Hussain, A. M. Jetten, and J. A. Fontana. 1993. Estrogen receptor-negative breast cancer cells transfected with the estrogen receptor exhibit increased RAR $\alpha$  gene expression and sensitivity to growth inhibition by retinoic acid. *J. Cell. Biochem.* 53:394-404.
55. Sheikh, M. S., Z. M. Shao, X. S. Li, M. Dawson, A. M. Jetten, S. Wu, B. A. Conley, M. Garcia, H. Rochefort, and J. A. Fontana. 1994. Retinoid-resistant estrogen receptor-negative human breast carcinoma cells transfected with retinoic acid receptor- $\alpha$  acquire sensitivity to growth inhibition by. *J. Biol. Chem.* 269:21440-21447.
56. Spanjaard, R. A., A. Sugawara, M. Ikeda, and W. W. Chin. 1995. Evidence that retinoid X receptors mediate retinoid-dependent transcriptional activation of the retinoic acid receptor  $\beta$  gene in S91 melanoma cells. *J. Biol. Chem.* 270:17429-17436.
57. Sucov, H. M., K. K. Murakami, and R. M. Evans. 1990. Characterization of an autoregulated response element in the mouse retinoic acid receptor type  $\beta$  gene. *Proc. Natl. Acad. Sci. USA* 87:5392-5396.
58. Swisshein, K., K. Ryan, X. Lee, H. C. Taou, M. Peacocke, and R. Sager. 1994. Down-regulation of retinoic acid receptor  $\beta$  in mammary carcinoma cell lines and its up-regulation in senescing normal mammary epithelial cells. *Cell Growth Differ.* 5:133-141.
59. Taneja, R., B. Roy, J.-L. Plassat, C. F. Zusl, J. Ostrowski, P. R. Reczek, and P. Chambon. 1996. Cell-type and promoter-context dependent retinoic acid receptor (RAR) redundancies for *RAR $\beta$ 2* and *Hoxa-1* activation in F9 and P19 cells can be artefactually generated by gene knockouts. *Proc. Natl. Acad. Sci. USA* 93:6197-6202.
60. van der Burg, B., B.-J. M. van der Leede, L. Kwakkenbos-Isbrucker, S. Salverda, S. W. de Laat, and P. T. van der Saag. 1993. Retinoid acid resistance of estradiol-independent breast cancer cells coincides with diminished retinoic acid receptor function. *Mol. Cell Endocrinol.* 91:149-157.
61. van der Leede, B. J., G. E. Folkers, C. E. van den Brink, P. T. van der Saag, and B. van der Burg. 1995. Retinoic acid receptor  $\alpha$  isoform is induced by estradiol and confers retinoic acid sensitivity in human breast cancer cells. *Mol. Cell. Endocrinol.* 109:77-86.
62. Wetherall, N. T., and C. M. Taylor. 1986. The effects of retinoid treatment and antiestrogens on the growth of T47D human breast cancer cells. *Eur. J. Cancer Clin. Oncol.* 22:53-59.
63. Willy, P. J., K. Umesono, E. S. Ong, R. M. Evans, R. A. Heyman, and D. J. Mangelsdorf. 1995. LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Dev.* 9:1033-1045.
64. Wilson, T. E., T. J. Fahrner, M. Johnston, and J. Milbrandt. 1991. Identification of the DNA binding site for NGFI-B by genetic selection in yeast. *Science* 252:1296-1300.
65. Woronicz, J. D., B. Cainan, V. Ngo, and A. Winoto. 1994. Requirement for the orphan steroid receptor nur77 in apoptosis of T-cell hybridomas. *Nature* 367:277-281.
66. Wu, Q., Y. Li, R. Liu, A. Agadir, M.-O. Lee, Y. Liu, and X.-K. Zhang. 1997. Modulation of retinoic acid sensitivity in lung cancer cells by a dynamic balance of nur77 and COUP-TF orphan receptors and their heterodimerization. *EMBO J.* 16:1656-1669.
67. Zhang, X.-K., B. Hoffmann, P. B. Tran, G. Graupner, and M. Pfahl. 1992. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. *Nature* 355:441-446.
68. Zhang, X.-K., J. Lehmann, B. Hoffmann, M. I. Dawson, J. Cameron, G. Graupner, T. Hermann, P. Tran, and M. Pfahl. 1992. Homodimer formation of retinoid X receptor induced by 9-cis retinoic acid. *Nature* 358:587-591.
69. Zhang, X.-K., Y. Liu, M. O. Lee, and M. Pfahl. 1994. A specific defect in the retinoic acid receptor associated with human lung cancer cell lines. *Cancer Res.* 54:5663-5669.
70. Zhang, X.-K., and M. Pfahl. 1993. Hetero- and homodimeric receptors in thyroid hormone and vitamin A action. *Receptor* 3:183-191.
71. Zhang, X.-K., G. Salbert, M.-O. Lee, and M. Pfahl. 1994. Mutations that alter ligand-induced switches and dimerization activities in the retinoid X receptor. *Mol. Cell. Biol.* 14:4311-4323.
72. Zhao, Z., Z. P. Zhang, D. R. Soprano, and K. J. Soprano. 1995. Effect of 9-cis-retinoic acid on growth and RXR expression in human breast cancer cells. *Exp. Cell Res.* 219:555-561.

## **Interaction of BAG-1 with Retinoic Acid Receptor and its Inhibition of Retinoic Acid-induced Apoptosis in Cancer Cells**

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## Abstract

**BAG-1** (also known as RAP46) is an anti-apoptotic protein, which has been shown previously to interact with a number of nuclear hormone receptors, including receptors for glucocorticoid, estrogen and thyroid hormone. We show here that BAG-1 also interacts with retinoic acid receptor (RAR). Gel retardation assays demonstrated that *in vitro* translated BAG-1 protein could effectively inhibit the binding of RAR but not retinoid X receptor (RXR) to a number of RA response elements. A glutathione S-transferase (GST)-BAG-1 fusion protein also specifically bound RAR but not RXR. Interaction of BAG-1 and RAR could also be demonstrated by yeast two-hybrid assays. In transient transfection assays, co-transfection of BAG-1 expression plasmid inhibited the transactivation activity of RAR/RXR heterodimers, but not RXR/RXR homodimers. When stably expressed in both MCF-7 and ZR-75-1 breast cancer cell lines, BAG-1 suppressed retinoic acid (RA)-induced growth inhibition and apoptosis. In addition, RA-induced suppression of Bcl-2 expression was abrogated by over-expression of BAG-1. These results demonstrate that BAG-1 can regulate retinoid activities through its interaction with RAR, and suggest that elevated levels of BAG-1 protein could potentially contribute to retinoid resistance in cancer cells.

## Introduction

Development of a multicellular organism requires tightly regulated cellular processes, such as proliferation, differentiation and cell death. Failure to maintain the balance among these fundamental and mechanistically related processes may result in abnormal cell growth, as seen in cancer cells where cell death is often inhibited (White, 1996; Reed, 1994). Retinoids, a group of natural and synthetic vitamin A derivatives, are currently used to treat epithelial cancer and promyelocytic leukemia and are being evaluated for prevention and therapy of other human cancers (Gudas *et al.*, 1994; Hong and Itri, 1994). The anti-cancer effects of retinoids were originally considered to be mainly due to its inhibition of cell proliferation and induction of cell differentiation. An increasing body of knowledge has now suggested that induction of apoptosis is another important mechanism by which retinoids exert their preventive and therapeutic effects. Retinoids alone or in combination with other stimuli induce apoptosis during normal development and in different types of cancer cells *in vitro*, including breast cancer (Liu *et al.*, 1996; Wu *et al.*, 1997a), lung cancer (Li *et al.*, 1997), prostate cancer (Anzick *et al.*, 1997), ovarian cancer (Chao *et al.*, 1997), and neuroblastoma (Ponzoni *et al.*, 1995). Induction of apoptosis of acute promyelocytic leukemia (APL) cells by retinoid acid (RA) may contribute to the therapeutic effect of RA on APL patients (Elstner *et al.*, 1996). How retinoid-induced apoptosis is regulated remains largely unknown.

The effects of retinoids are mainly mediated by two classes of nuclear receptors, the RA receptors (RARs) and retinoid X receptors (RXRs). RARs and RXRs are encoded by three distinct genes ( $\alpha$ ,  $\beta$ , and  $\gamma$ ), and are members of the steroid/thyroid/retinoid hormone receptor superfamily that function as ligand-activated transcription factors (Zhang and Pfahl, 1993; Kastner *et al.*, 1995; Mangelsdorf and Evans, 1995). 9-cis RA is a high affinity ligand for both RARs and RXRs, whereas all-trans-RA (*trans*-RA) is a ligand for only RARs. RARs and RXRs

primarily function as RXR/RAR heterodimers that bind to a variety of RA-response elements (RAREs) and regulate their transactivation activities.

Regulation of gene expression either positively or negatively by nuclear hormone receptors is modulated by additional cofactors that appear to provide a direct link to the core transcriptional machinery and to modulate chromatin structure (Horwitz *et al.*, 1996). Some of these cofactors (coactivators), such as steroid receptor coactivator 1 (SRC-1) (Onate *et al.*, 1995), SUG-1 (Lee *et al.*, 1995), TIF-1 (Ledouarin *et al.*, 1995), RIP-140 (Cavailles *et al.*, 1995), TIF-2 (Voegel *et al.*, 1996), glucocorticoid receptor interacting protein 1 (GRIP-1 (Hong *et al.*, 1996), p160 (Halachmi *et al.*, 1994), CREB-binding protein (CBP) (Kamei *et al.*, 1996), AIB1 (Anzick *et al.*, 1997), and ACTR (Chen *et al.*, 1997), enhance transactivation by several nuclear receptors in the presence of their cognate ligands, whereas others (corepressors), such as N-CoR (nuclear receptor corepressor) (Horlein *et al.*, 1995) and the related factor SMRT (silencing mediator of retinoic acid and thyroid hormone receptors) (Chen and Evans, 1995), are necessary for unliganded receptors to silence the activity of target promoters, and are dissociated upon binding of ligand to the receptors. In addition to receptor coactivators and corepressors, a number of other cellular proteins, such as AP-1, have been implicated in the regulation of nuclear hormone receptor activity, probably through their interaction with receptors (Pfahl, 1993).

The involvement of retinoid receptors in retinoid-induced apoptosis has been demonstrated in several studies. Expression of RAR $\beta$  may be involved in the apoptosis of mesenchyme of the interdigital regions during mouse limb development (Kochhar *et al.*, 1993), and was shown to contribute to RA-induced apoptosis of breast cancer (Liu *et al.*, 1996) and lung cancer (Li *et al.*, 1997) cells, whereas activation of RXR was essential for RA-induced HL-60 cell apoptosis (Nagy *et al.*, 1995). In 4-HPR-induced apoptosis, activation of RAR $\gamma$  may be

involved (Fanjul *et al.*, 1996b; Fanjul *et al.*, 1996a). Regulation of activation-induced apoptosis of T-cells by 9-cis RA required activation of both RARs and RXRs (Yang *et al.*, 1995).

Although much interest has been directed to the role of retinoid-induced apoptosis in both physiological and pathological processes, very little is known regarding the underlying molecular mechanism. It is believed that apoptosis, once triggered, proceeds through a central death pathway in which specific cellular proteases and endonucleases are activated (White, 1996; Steller, 1995; Reed, 1994). Members of the Bcl-2 family play an important role in the regulation of the central death pathway. Bcl-2 can suppress induction of apoptosis in many systems, whereas Bax promotes apoptosis. Interestingly, some retinoids can down-regulate expression of Bcl-2 under certain circumstances (Elstner *et al.*, 1996).

In addition to Bax, several other proteins that modulate Bcl-2 activity in either a positive or negative way by interacting with Bcl-2 have been described (White, 1996; Steller, 1995; Reed, 1994). One of these genes, BAG-1 (for Bcl-2-associated Anti-death Gene 1), was cloned from a murine embryo cDNA library using a protein-protein interaction technique (Takayama *et al.*, 1995). Co-expression of BAG-1 and Bcl-2 in Jurkat lymphoid cells, NIH 3T3 fibroblasts, and melanoma cells promoted the survival of these cells in response to a variety of apoptotic stimuli (Takayama *et al.*, 1995; Clevenger *et al.*, 1997; Takaoka *et al.*, 1997). In addition to Bcl-2, BAG-1 also interacts with Raf-1 (Wang *et al.*, 1996), and can activate this kinase through a Ras-independent mechanism. Furthermore, BAG-1 can interact with hepatocyte growth factor (HGF) receptor and with platelet-derived growth factor (PDGF) receptor and enhance the ability of these receptors to transduce signals for cell survival (Bardelli *et al.*, 1996). These observations suggest that BAG-1 may function as an adaptor to mediate the interaction between survival factors and apoptotic machinery, and may also play a role in regulating cellular proliferation.

Recently, it has been shown that BAG-1 binds tightly to Hsp70/Hsc70-family proteins and modulates their chaperone activity (Takayama *et al.*, 1997a). Thus, the ability of BAG-1 to alter the activities of diverse groups of proteins involved in cell growth control can probably be attributed to its effects of Hsp70/Hsc70 proteins.

Interestingly, the human BAG-1 homolog (also known as RAP46) was cloned from a human liver cDNA library by virtue of its interaction with the glucocorticoid receptor (Zeiner and Gehring, 1995). In vitro, RAP46 interacts with a number of nuclear hormone receptors, including estrogen receptor (ER) and thyroid hormone receptor (TR) (Zeiner and Gehring, 1995). Since molecular chaperones are known to play an important role in controlling the activity of many members of the steroid/thyroid/retinoid receptor family (Pratt and Toft, 1997), it is possible that BAG-1 could alter the function of these transcriptional regulators. Prior to this report, however, it was unknown whether BAG-1 regulates the activities of the nuclear hormone receptors and whether BAG-1 interacts with retinoid receptors.

Here we demonstrate that BAG-1 interacts with the RAR but not the RXR both in vitro and in vivo. GST pull-down and the yeast two-hybrid assays demonstrated that BAG-1 could directly interact with RAR but not RXR. Moreover, BAG-1 can inhibit RAR/RXR heterodimer DNA binding and suppresses RA-induced transactivation activity of RARs on various RAREs. Over-expression of MCF-7 and ZR-75-1 breast cancer cells inhibited RA-induced growth inhibition and apoptosis. In addition, RA-induced inhibition of Bcl-2 expression was abrogated by over-expression of BAG-1. Taken together, our results demonstrate that BAG-1 can physically interact with RARs and is an important component of the retinoid response pathway. The findings suggest that this protein-protein interaction may play an important role in the regulation of retinoid-induced growth inhibition and apoptotic processes, potentially contributing to retinoid resistance in cancer.

## Results

BAG-1 was shown to interact with a number of nuclear hormone receptors, including GR, ER and TR (Zeiner and Gehring, 1995). We investigated whether BAG-1 could also interact with retinoid receptors by studying the effect of BAG-1 protein on binding of retinoid receptors to their target DNA sequences. In vitro synthesized RAR and RXR formed a strong RAR/RXR heterodimer complex with the oligonucleotide TREP<sub>1</sub> as described previously (Zhang *et al.*, 1992a). When increasing amounts of in vitro synthesized BAG-1 protein were incubated with RAR and RXR, the binding of RAR/RXR heterodimers was inhibited in a BAG-1 concentration-dependent manner (Figure 1a). At a five-molar excess of BAG-1 protein relative to RAR/RXR, the binding was almost completely inhibited. The effect of BAG-1 on RAR/RXR binding was specific because similar excess amounts of ER did not show any effect. BAG-1 also effectively inhibited the binding of TR/RXR to the same TREP<sub>1</sub> probe (Figure 1b), consistent with a prior report that BAG-1 can interact with TR (Zeiner and Gehring, 1995). To study whether the inhibitory effect of BAG-1 on RAR/RXR heterodimer binding is specific to the TREP<sub>1</sub>, we used another RA responsive element ( $\beta$ RARE), that is derived from the RAR $\beta$  promoter (Hoffmann *et al.*, 1990). As shown in Figure 1c, binding of RAR/RXR on the  $\beta$ RARE was also inhibited by the addition of BAG-1 protein. Similar results were obtained with other RAREs, including CRBPI-RARE and ApoAI-RARE (data not shown). Thus, BAG-1 mediated inhibition of RAR/RXR binding to target DNA sequences is independent of the specific RAREs.

We next determined whether inhibition of RAR/RXR heterodimer binding by BAG-1 is due to interaction of BAG-1 with RAR or with RXR. Since in vitro synthesized RAR or RXR alone do not bind efficiently to RARE, we used bacterially expressed RAR or RXR proteins. Bacterially expressed RAR $\gamma$  or RXR $\alpha$  alone bound effectively to the TREP<sub>1</sub> (Figure 1d). When a five-fold molar excess of BAG-1 protein was added, the binding of RAR $\gamma$  was significantly

inhibited. In contrast, binding of RXR $\alpha$  was not inhibited by BAG-1 (Figure 1d). These results suggest that inhibition of RAR/RXR or TR/RXR heterodimer binding to target DNA sequences by BAG-1 is likely due to interaction between BAG-1 and RAR or BAG-1 and TR, but not to interaction between BAG-1 and RXR.

To further study the interaction between BAG-1 and RAR, we used an in vitro GST pull-down assay. A GST-BAG-1 fusion protein was expressed in bacteria, and immobilized on glutathione-sepharose beads. The beads were then incubated with in vitro synthesized  $^{35}$ S-labeled RAR or RXR protein. After extensive washing, the mixtures were analyzed on a SDS-polyacrylamide gel. In comparison to the input lane, significant amounts of labeled RAR were retained by GST-BAG-1-Sepharose beads but not by GST control beads. Under the same conditions, little or no binding of RXR to GST-BAG-1 was observed. For control, Bcl-2, a known BAG-1 interacting protein (Takayama *et al.*, 1995), bound strongly to GST-BAG-1 beads. These data further demonstrate that BAG-1 interacts with RAR but not with RXR. We also employed a BAG-1 mutant protein in which the last 47 amino acid residues are deleted from its C-terminal end (Takayama *et al.*, 1997a). This mutant protein (BAG-1/C) can interact with Bcl-2 but not with Hsc70. Interestingly, labeled RAR but not RXR was also retained by the mutant BAG-1, thus implying that the interaction of BAG-1 with RAR is independent of binding to Hsc70.

Interaction between RAR and BAG-1 was also evaluated by the two-hybrid assay in yeast (Figure 3). BAG-1 cDNA was cloned in frame into the yeast expression vector pGAD424 that contains the Gal4 activation domain, and RAR $\gamma$  was cloned into the yeast expression vector pGBT11 that contains the Gal4 DNA-binding domain. The resulting vectors, pGAD424/BAG-1 and pGBT11/RAR $\gamma$ , were co-transformed into Y190 yeast cells containing a LacZ reporter with Gal4 binding site. RXR $\alpha$  was also cloned into pGBT9 to evaluate its interaction with BAG-1. In addition, interaction between RAR $\gamma$  and RXR $\alpha$  was studied for comparison. Figure 3 shows that co-transformation of BAG-1 and RAR $\gamma$  strongly activated the reporter in  $\beta$ -gal filter assays, while

co-transformation of BAG-1 and RXR $\alpha$  did not. Interaction between BAG-1 and RAR $\gamma$  was specific because co-transformation of either BAG-1 with the pGBT11 empty vector or RAR $\gamma$  with the pGAD424 empty vector did not induce the LacZ reporter gene. Thus, RAR and BAG-1 can also interact in intact cells *in vivo*.

To further examine the BAG-1/RAR interaction, we studied the effects of BAG-1 on RAR mediated transactivation of a number of RARE-containing reporter plasmids by transient transfection assays. CV-1 cells were chosen for these studies because they are known to contain undetectable levels of nuclear hormone receptors. When CV-1 cells were transiently transfected with RAR $\alpha$  expression vector together with either TREpal-tk-CAT (Figure 4a) or  $\beta$ RARE-tk-CAT (Figure 4b) and then treated with *trans*-RA, transcription of the reporter genes was strongly induced. The *trans*-RA-induced reporter gene activity was markedly inhibited when cells were co-transfected with BAG-1 expression plasmid. Moreover, this inhibition was BAG-1 concentration dependent (Figure 4a). The effect is specific to BAG-1 since co-transfection of similar amounts of empty expression vector (pcDNA3) did not inhibit *trans*-RA-induced gene expression. The RAR family of retinoid-responsive transcription factors includes RAR $\alpha$ , RAR $\beta$ , and RAR $\gamma$  (Zhang and Pfahl, 1993; Kastner *et al.*, 1995; Mangelsdorf and Evans, 1995). To examine the effects of BAG-1 on RAR $\beta$ , CV-1 cells were co-transfected with a RAR $\beta$  expression plasmid and TREpal-tk-CAT reporter gene with or without BAG-1 encoding plasmid. Transfection of the RAR $\beta$  expression vector significantly enhanced the reporter basal activity. A further increase in the TRE-tk-CAT expression was observed when cells were treated with *trans*-RA. When the BAG-1 expression vector was co-transfected, *trans*-RA-induced reporter activity was strongly inhibited. However, the *trans*-RA-independent RAR $\beta$ -induced basal reporter activity was not affected by BAG-1. This suggests that ligand-dependent RAR activity is more sensitive to the inhibitory effect of BAG-1. A similar result was also obtained when RAR $\gamma$  expression vector was used (data not shown). For comparison, we also studied the effects of BAG-1 on thyroid hormone (T<sub>3</sub>)-induced TR $\alpha$  activity using the TRE<sub>MHC</sub>-tk-CAT plasmid as a reporter (Hermann *et al.*, 1992). Again, we observed a

significant inhibitory effect of BAG-1 on TR $\alpha$  (Figure 4d), consistent with the ability of BAG-1 to bind the TR protein (Figure 1b; Zeiner and Gehring, 1995).

To determine the effect of BAG-1 on RXR activity, we co-transfected TREpal-tk-CAT reporter plasmid and a RXR $\alpha$  expression vector. When cells were treated with the RXR ligand 9-*cis*-RA, we observed a large increase in reporter gene activity due to induction of RXR $\alpha$  homodimer formation by 9-*cis*-RA (Zhang *et al.*, 1992b). This 9-*cis*-RA-induced transactivation by RXR $\alpha$  was not affected by co-transfection of BAG-1 expression vector (Figure 4e). This observation further supports our data showing that BAG-1 does not interact with RXR $\alpha$  in vitro (Figures 1d, 2 and 3)

The above data suggest that BAG-1 may function as a modulator of *trans*-RA-induced biological responses. We previously showed that *trans*-RA effectively inhibits the growth and induces apoptosis of some human breast cancer cell lines (Liu *et al.*, 1996). We, therefore, stably expressed BAG-1 into the human breast cancer cell lines MCF-7 and ZR-75-1, and examined its influence on *trans*-RA effects. MCF-7/BAG-1(#3), 75-1/BAG-1(#5) and 75-1/BAG-1(#6) are stably transfected clones that expressed high levels of the transfected BAG-1 plasmid (data not shown). As shown in Figure 5, *trans*-RA effectively inhibited the growth of parental MCF-7 and ZR-75-1 cells in a concentration-dependent manner, as described previously (Liu *et al.*, 1996). However, the BAG-1 over-expressing clones displayed resistance to the growth inhibitory effects of *trans*-RA. Indeed, the growth of the BAG-1 over-expressing MCF-7 cells, MCF-7/BAG-1 (#3), was even stimulated by *trans*-RA (Figure 5a). Though the growth of ZR-75-1-BAG-1 cells, 75-1/BAG-1 (#5) and 75-1/BAG-1 (#6), was not stimulated by *trans*-RA it was less inhibited by *trans*-RA compared to ZR-75-1 cells (Figure 5b). The resistance to *trans*-RA observed was specifically due to BAG-1 since clones stably transfected with the empty vector, MCF-7/neo and 75-1/neo, exhibited similar responses to *trans*-RA as that observed with the parental cell lines.

These data therefore demonstrate that BAG-1 partially abrogates the growth inhibitory effects of *trans*-RA on human breast cancer cells.

We next investigated the effects of BAG-1 on *trans*-RA-induced apoptosis of ZR-75-1 and MCF-7 cells using the terminal deoxynucleotidyl transferase (TdT) assay. Extensive DNA fragmentation was induced in the parental ZR-75-1 cells and ZR-75-1 cells transfected with the empty vector (ZR-75-1/neo) after treatment with  $10^{-6}$  M *trans*-RA for 2 days. In the experiment shown in Figure 6a, about 39% and 33% of the ZR-75-1 and ZR-75-1/neo underwent apoptosis, respectively. However, ZR-75-1/BAG-1(#5) and ZR-75-1/BAG-1 (#6) cells experienced much less DNA fragmentation under the same conditions, with only about 7% and 8% apoptotic cells, respectively. Similarly, treatment of MCF-7 and MCF-7/neo cells with *trans*-RA for two days resulted in 24% and 22% apoptotic cells, respectively, in this experiment (Figure 6b). In contrast, the apoptogenic effect of *trans*-RA was significantly reduced in MCF-7/BAG-1(#3) cells, with only about 8% of the cells undergoing apoptosis. Thus, over-expression of BAG-1 inhibits *trans*-RA-induced apoptosis in breast cancer cells.

Retinoids have been shown to down-regulate expression of the anti-apoptotic gene Bcl-2 in leukemia (Elstner *et al.*, 1996). We therefore studied the effect of BAG-1 on expression of Bcl-2 in MCF-7 cells. Bcl-2 was highly expressed in parental MCF-7 cells as determined by immunoblotting. *Trans*-RA induced substantial decreases in the level of Bcl-2 protein in parental MCF-7 cells, with exposure to  $10^{-6}$  M *trans*-RA for 1 day reducing Bcl-2 levels by 80%. Levels of Bcl-2 were further reduced after prolonged *trans*-RA treatment in parental MCF-7 cells. In MCF-7/BAG-1#3 stable transfectant, however, treatment of *trans*-RA for 1 or 2 days had little or no effect on Bcl-2 expression (Figure 7). These data suggest that BAG-1 may inhibit *trans*-RA-induced apoptosis at least in part through its effects on *trans*-RA-regulated genes which are involved in apoptosis regulation, such as Bcl-2.

## Discussion

Previously, it was reported that BAG-1 (RAP60) can bind to several steroid hormone receptors (Zeiner and Gehring, 1995). Though retinoid receptors were not examined, BAG-1 was shown to interact in vitro with GR, ER, PR, AR and TR (Zeiner and Gehring, 1995). However, the biological effects of interactions of BAG-1 with these receptors are unknown. In this report, we show that BAG-1 can bind and inhibit the function of RARs. We also present evidence that BAG-1 can similarly prevent TR binding to its recognition sites in DNA and interfere with its ability to trans-activate target genes. Using gel retardation assays, we observed that BAG-1 could inhibit binding of RAR/RXR heterodimers to several RAREs (Figure 1). In GST-pull down assay, we found that BAG-1 could directly interact with RAR in solution (Figure 2). By using yeast two-hybrid assays (Figure 3), we showed that the BAG-1/RAR interaction could occur *in vivo*. Moreover, a functional interaction was demonstrated by our observation that co-transfection of BAG-1 could inhibit *trans*-RA-induced RAR transactivation of several RARE-containing reporter plasmids in CV-1 cells (Figure 4). Thus, the interaction of BAG-1 with steroid hormone receptors can be extended to RAR. However, BAG-1 does not interact with all nuclear hormone receptors, as shown here by the failure of BAG-1 to bind to and modulate the activity of RXR (Figures 1d, 2, 3 and 4e).

Although our studies clearly demonstrate the interaction between BAG-1 and RAR, the role of retinoid ligands in the interaction is unclear. In vitro, we did not see any effect of *trans*-RA on binding of BAG-1 to RAR (data not shown). However, based on co-transfection assay, it appears that only *trans*-RA-induced RAR $\beta$  activity was inhibited by BAG-1, whereas *trans*-RA-independent activity of RAR $\beta$  was unaffected (Figure 4c). This suggests that BAG-1/RAR interaction may be *trans*-RA dependent.

Activation or repression of gene transcription by nuclear hormone receptors requires their interaction with multiple cellular co-regulatory factors. These include receptor co-activators, that may exert their effect on receptor trans-activation activity by mediating transcription-initiation complex formation or affecting chromatin structure (Horwitz *et al.*, 1996). A CREB-binding protein, CBP, that interacts with phosphorylated CREB and that is required for activation of both CREB and AP-1, has been reported to function as a co-activator of various nuclear hormone receptors, including RARs (Kamei *et al.*, 1996). Another group of regulatory proteins are receptor co-repressors, that bind to receptors in the absence of ligand and actively repress target gene transcription by impairing the activity of the basal transcription machinery (Horlein *et al.*, 1995). BAG-1 appears to function differently from receptor co-activators or co-repressors. It does not induce ligand-dependent gene activation nor does it cause repression of target gene transcription in the absence of ligand (Figure 4a). Instead, interaction of RAR and BAG-1 resulted in inhibition of RAR DNA binding (Figure 1) and *trans*-RA-induced RAR trans-activation activities (Figure 4). Thus, it may function as a modulator of RAR activities through the mechanisms that resemble the effect of AP-1, which was previously shown to inhibit *trans*-RA-induced RAR activity by preventing RAR binding to target DNA sequences in promoters (Pfahl, 1993).

The mechanism by which BAG-1 inhibits RAR binding to DNA and trans-activation activity remains to be elucidated. Recently, however, BAG-1 was reported to bind tightly with Hsp70/Hsc70-family proteins and modulate their activity (Takayama *et al.*, 1997a). Though the role of molecular chaperones in transcriptional activation by retinoid receptors remains controversial (Dalman *et al.*, 1991; Holley and Yamamoto, 1995), Hsp70/Hsc70 and other heat shock proteins are known to participate in important ways in the regulation of several other steroid hormone receptors (Pratt and Toft, 1997). It is therefore tempting to speculate that BAG-1 influences RAR activity through Hsp70/Hsc70-mediated conformational changes that prevent it from binding DNA and trans-activating retinoid responsive target genes. Interestingly, a deletion mutant of BAG-1 lacking its C-terminal 47 amino acids was capable of binding to RAR *in vitro*.

but fail to bind to Hsc70 (Figure 2). Thus, the domains in BAG-1 that are required for interactions with RAR and Hsc70 appear to be separable. Unfortunately, when expressed in mammalian cells, the BAG-1 mutant protein was unstable, precluding functional evaluation of its effects on RAR-mediated gene expression. Thus, the relevance of BAG-1 interactions with Hsp70-family proteins to its function as inhibitor of RAR remains to be determined.

One of the interesting features of BAG-1 is its ability to promote cell survival (Takayama *et al.*, 1995; Takaoka *et al.*, 1997; Clevenger *et al.*, 1997). The effect was previously attributed to its interaction with Bcl-2 (Takayama *et al.*, 1995). In addition, BAG-1 was also reported to interact with PDGF and HGF receptors, enhancing their ability to transduce signals that promote cell survival (Bardelli *et al.*, 1996). BAG-1 can also bind and activate the kinase Raf-1, which has been implicated in both mitogenesis and cell survival (Wang *et al.*, 1996). Our observation that stable over-expression of BAG-1 inhibited *trans*-RA-induced apoptosis (Figure 6) and prevented *trans*-RA-induced down-regulation of Bcl-2 expression (Figure 7) suggests that interaction with RAR may represent another mechanism by which BAG-1 promotes cell survival. These observations also suggest that over-expression of BAG-1 may contribute to retinoid resistance in certain malignancies. Taken together with recent observations of elevations in BAG-1 protein levels in breast and prostate cancers (Takayama *et al.*, 1997b), therefore, these diverse functions of BAG-1 suggest that it is an important regulator of cell survival and growth which may contribute in multiple ways to tumorigenesis and resistance to therapy.

## Materials and Methods

### Cell culture

Monkey kidney CV-1 cells and breast cancer MCF-7 cells were grown in DMEM medium supplemented with 10% fetal calf serum (FCS), and ZR-75-1 breast cancer cells were maintained in RPMI 1640 medium supplemented with 10% FCS.

### **Growth inhibition assay**

Cells were seeded at 1,000-2,000 cells per well in 96-well plates and treated 24 h later with various concentrations of *trans*-RA for 7 days. The control cells received vehicle (ethanol). Media and *trans*-RA were changed every 48 h. Relative viable cell number was determined using the MTT assay in which the capacity of cells to convert a tetrazolium salt to a blue formazan product was measured (Promega, Madison, WI) (Wu *et al.*, 1997b).

### **Apoptosis analysis**

For the terminal deoxyribonucleotidyl transferase (TdT) assay (Liu *et al.*, 1996), cells were treated with or without  $10^{-6}$  M *trans*-RA. After 48 h, cells were trypsinized, washed with PBS, fixed in 1% formaldehyde in PBS, washed with PBS, resuspended in 70% ice-cold ethanol, and stored at -20°C overnight. Cells were then labeled with biotin-16-dUTP by terminal transferase and stained with avidin-FITC (Boehringer Mannheim). Fluorescently labeled cells were analyzed using a FACScater-Plus. Representative histograms are shown.

### **Antibodies and Immunoblotting**

Cells were lysed in 150 mM NaCl, 10 mM Tris [p47.4], 5mM EDTA, 1 % Triton X-100 and protease inhibitors phenylmethylsulphonyl fluroide (PMSF), aprotinin, leupeptins and pepstatin. Equal amounts of lysates (50 µg) were boiled in SDS sample buffer, resolved by SDS-polyacrylamide gel (12.5%) electrophoresis, and transferred onto nitrocellulose. After transfer, the membranes were blocked in TBST (50 mM Tris [pH7.5], 150 mM NaCl, 0.1% Tween 20) containing rabbit anti-Bcl-2 serum. The membranes were then washed three times with TBST and then incubated for 1 h at room temperature in TBST containing horseradish peroxidase-linked anti-rabbit immunoglobulin. After three washes in TBST, immunoreactive products were detected by chemiluminescence with an enhanced chemiluminescence system (ECL, Amersham).

### **Transient and stable transfection assay.**

For CV-1 cells,  $1 \times 10^5$  cells were plated per well in a 24-well plates 16-24 h before transfection as described previously (Zhang *et al.*, 1992a). For ZR-75-1 cells,  $5 \times 10^5$  cells were seeded in six-well culture plates. A modified calcium phosphate precipitation procedure was used for transient transfection (Zhang *et al.*, 1992a). For CV-1 cells, 100 ng of reporter plasmid, 150 ng  $\beta$ -galactosidase expression vector (pCH 110, Pharmacia), and various amounts of BAG-1 expression vector were mixed with carrier DNA (pBluescript) to 1,000 ng of total DNA per well. CAT activities were normalized for transfection efficiency using  $\beta$ -gal activity. Reporter plasmids  $\beta$ RARE-tk-CAT, TREpal-tk-CAT and TRE<sub>MHC</sub>-tk-CAT have been previously described (Zhang *et al.*, 1992a; Hoffmann *et al.*, 1990; Hermann *et al.*, 1992). For stable transfection, the pRc/CMV-BAG-1 plasmid (Takayama *et al.*, 1995) was stably transfected into MCF-7 or ZR-75-1 cells using calcium phosphate precipitation method, followed by selection using G418 (GIBCO BRL, Grand Island, NY) as described (Liu *et al.*, 1996).

### **Preparation of receptor and BAG-1 protein**

cDNAs for RAR $\alpha$ , RXR $\alpha$ , TR $\alpha$ , ER and BAG-1 cloned into pBluescript (Stratagene) were transcribed by using T<sub>7</sub> and T<sub>3</sub> RNA polymerase, and the transcripts were translated in the rabbit reticulocyte lysate system (Promega) as described previously (Zhang *et al.*, 1992a). The relative amounts of the translated proteins were determined by separating the <sup>35</sup>S-methionine labeled proteins on sodium dodecyl sulfate (SDS)-polyacrylamide gels, quantitating the amount of incorporated radioactivity, and normalizing it relative to the content of methionine residues in each protein. To synthesize receptor fusion protein, RAR $\gamma$  or RXR $\alpha$  cDNAs were cloned in frame into the bacterial expression vector pGex.2T (Pharmacia) as described (Zhang *et al.*, 1992a). These glutathione S-transferase (GST) fusion proteins were expressed in bacteria, and purified on a prepacked glutathione Sepharose 4B column (Pharmacia). The preparation and purification of GST-BAG-1 and the GST-BAG-1 ( $\Delta$ 172-218) fusion proteins has been described (Takayama *et al.*, 1997a).

### **Gel retardation assay**

In vitro synthesized or bacterially expressed receptor proteins were incubated with  $^{32}\text{P}$ -labeled oligonucleotides in a 20  $\mu\text{l}$  reaction mixture containing 10 mM HEPES buffer (pH7.9), 50 mM KCl, 1 mM DTT, 2.5 mM MgCl<sub>2</sub>, 10% glycerol, and 1  $\mu\text{g}$  poly (dI-dC) at 25°C for 20 min. The reaction mixture was then loaded into a 5% non-denaturing polyacrylamide gel containing 0.5X TBE (1 x TBE is 0.089 M Tris-borate; 0.089 M boric acid, and 0.002 M EDTA). When binding of RAR/RXR heterodimers was analyzed, RAR and RXR proteins were preincubated at room temperature for 10 min. To analyze the effect of BAG-1 protein, in vitro synthesized BAG-1 protein was preincubated with receptor protein at room temperature for 10 min prior to gel retardation assay. The oligonucleotides used for gel retardation assays were  $\beta$ RARE (TGTAGGGTTCACCGAAAGTTCAAGTC) (Hoffmann *et al.*, 1990) and TREpal (TGAGGTCATGACCTGA) (Zhang *et al.*, 1992a).

### **GST pull-down assay**

To analyze the interaction between BAG-1 and RAR, GST-BAG-1 fusion protein was immobilized on glutathione-Sepharose beads, as described (Wu *et al.*, 1997b). As a control, GST prepared under the same conditions was also immobilized. The beads were preincubated with BSA (1 mg/ml) at room temperature for 5 min.  $^{35}\text{S}$ -labeled in vitro synthesized receptor proteins (2 to 5  $\mu\text{l}$ , depending on translation efficiency), Bcl-2 or Hsc70 were then added to the beads. The beads were then continuously rocked for 1 h at 4°C in a final volume of 200  $\mu\text{l}$  in EBC buffer (140 mM NaCl, 0.5% NP40, 100 mM NaF, 200  $\mu\text{M}$  Sodium Orthovanadate, and 50 mM Tris, pH8.0). After washing five times with NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], 0.5% NP40), the bound proteins were analyzed by SDS-PAGE.

### **Two-hybrid assay.**

For the yeast two-hybrid assay, the yeast two-hybrid system from Clontech Inc. (Palo Alto, CA) was used (Wu *et al.*, 1997b). BAG-1 cDNA was cloned into the yeast expression vector

pGAD424 to generate an in-frame fusion with the Gal4 activation domain. RAR $\gamma$  or RXR $\alpha$  cDNAs were cloned into pGBT11 or pGBT9, respectively, to produce an in-frame fusion with Gal4 DNA binding domain. RAR $\gamma$  was also cloned into pGAD426 that contains Gal4 activation domain in order to study the interaction between RAR $\gamma$  and RXR $\alpha$ . The yeast reporter strain Y190 containing a LacZ reporter plasmid with Gal4 binding sites was used for transformation.  $\beta$ -galactosidase activity was determined following the conditions provided by the manufacturer.

### Acknowledgments

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### References

- Anzick,S.L., Kononen,J., Walker,R.L., Azorsa,D.O., Tanner,M.M., Guan,X.-Y., Sauter,G., Kallioniemi,O.-P., Trent,J.M. and Meltzer,P.S. (1997) AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science*, **277**, 965-968.
- Bardelli,A., Longati,P., Albero,D., Goruppi,S., Schneider,C., Ponzetto,C. and Comoglio,P.M. (1996) HGF receptor associates with the anti-apoptotic protein BAG-1 and prevents cell death. *EMBO J.*, **15**, 6205-6212.
- Cavailles,V., Dauvois,S., Horset,F.L., Lopez,G., Hoare,S., Kushner,P.J. and Parker,M.G. (1995) Nuclear factor RIP 140 modulates transcriptional activation by the estrogen receptor. *EMBO J.*, **14**, 3741-3751.
- Chao,W.R., Hobbs,P.D., Jong,L., Zhang,X.-k., Zheng,Y., Wu,Q., Shroot,B. and Dawson,M.I. (1997) Effects of receptor class- and subtype-selective retinoids and an apoptosis-inducing retinoid on the adherent growth of the NIH:OVCAR-3 ovarian cancer cell line in culture. *Cancer Lett.*, **115**, 1-7.
- Chen,H., Lin,R.J., Schlitz,R.L., Chakravarti,D., Nash,A., Nagy,Y., Privalsky,M.L., Nakatani,Y. and Evans,R.M. (1997) Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell*, **90**, 569-580.
- Chen,J.D. and Evans,R.M. (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature*, **37**, 454-457.

Clevenger,C.V., Thickman,K., Ngo,W., Chang,W.-P., Takayama,S. and Reed,J.C. (1997) Role of Bag-1 in the survival and proliferation of the cytokine-dependent lymphocyte lines, Ba/F3 and Nb2. *Mol. Endocrinol.*, **11**, 608-618.

Dalman,F.C., Sturzenbecker, L.J., Levin,A.A., Luca,D.A., Perdew,G.H., Petkovitch,M., Chambon,P., Grippo,J.F. and Pratt,W.B. (1991) Retinoic acid receptor belongs to a subclass of nuclear receptors that do not form "docking" complexes with hsp90. *Biochemistry*, **30**, 5605-5608.

Elstner,E., Linker-Israeli,M., Umie,T., Le,J., Grillier,I., Said,J., Shintaku,I.P., Krajewski,S., Reed,J.C., Binderup,L. and Koeffler,H.P. (1996) Combination of a potent 20-*epi*-vitamin D3 ananlogue (KH 1060) with 9-*cis*-retinoic acid irreversibly inhibits clonal growth, decreases bcl-2 expression, and induces apoptosis in HL-60 leukemic cells. *Cancer Res.*, **56**, 3570-3576.

Fanjul,A.N., Bouterfa,H., Dawson,M. and Pfahl,M. (1996a) Potential role for retinoic acid receptor- $\gamma$  in the inhibition of breast cancer cells by selective retinoids and interferons. *Cancer Res.*, **56**, 1571-1577.

Fanjul,A.N., Delia,D., Pierotti,M.A., Rideout,D., Qiu,J. and Pfahl,M. (1996b) Hydroxyphenyl retinamide is a highly selective activator of retinoid receptors. *J. Biol. Chem.*, **271**, 22441-22446.

Gudas, L. J., Sporn, M. B. & Roberts, A. B. (1994) in *The Retinoids*, eds. Sporn, M. B., Roberts, A. B. & Goodman, D. S. (Raven Press, New York), pp. 443-520.

Halachmi,S., Marden,E., Martin,G., MacKay,H., Abbondanza,C. and Brown,M. (1994) Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. *Science*, **264**, 1455-1458.

Hermann,T., Hoffmann,B., Zhang,X.-k., Tran,P. and Pfahl,M. (1992) Heterodimeric receptor complexes determine 3,5,3'-Triiodothyronine and retinoid signaling specificities. *Mol. Endocrinol.*, **6**, 1153-1162.

Hoffmann,B., Lehmann,J.M., Zhang,X.-k., Hermann,T., Graupner,G. and Pfahl,M. (1990) A retinoic acid receptor-specific element controls the retinoic acid receptor- $\beta$  promoter. *Mol. Endocrinol.*, **4**, 1734-1743.

Holley,S.J. and Yamamoto,K.R. (1995) A role for Hsp90 in retinoid receptor signal transduction. *Mol. Biol. Cell.*, **6**, 1833-1842.

Hong,H., Kohli,K., Trivedi,A., Johnson,D.L. and Stallcup,M.R.G. (1996) RIP1, A novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. *Proc. Natl. Acad. Sci. USA*, **93**, 4948-4952.

Hong, W. K. and Itri, L. M. (1994) in *The Retinoids*, eds. Sporn, M. B., Roberts, A. B. & Goodman, D. S. (Raven Press, New York), pp. 597-630.

Horlein,A.J., Naar,A.M., Heinzel,T., Torchia,J., Gloss,B., Kurokawa,R., Ryan,A., Kamei,Y., Soderstrom,M., Glass,C.K. and Rosenfeld,M.G. (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature*, **37**, 454-457.

Horwitz,K.B., Jackson,T.A., Bain,D.L., Richer,J.K., Takimoto,G.S. and Tung,L. (1996) Nuclear receptor coactivators and corepressors. *Mol. Endocrinol.*, **10**, 1167-1177.

- Kamei,Y., Xu,L., Heinzel,T., Torchia,J., Kurokawa,R., Gloss,B., Lin,S.-C., Heyman,R.A., Rose,D.W., Glass,C.K. and Rosenfeld,M.G. (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell*, **85**, 403-414.
- Kastner,P., Mark,M. and Chambon,P. (1995) Nonsteroid nuclear receptors: What are genetic studies telling us about their role in real life? *Cell*, **83**, 859-869.
- Kochhar,D.M., Jiang,H., Harnish,D.C. and Soprano,D.R. (1993) Evidence that retinoic acid-induced apoptosis in the mouse limb bud core gene-mediated. *Prog. Clin. Biol. Res.*, **383B**, 815-825.
- Ledouarin,B., Zechel,C., Garnier,J.M., Lutz,Y., Tora,L., Pierrat,B., Heery,D., Gronemeyer,H., Chambon,P. and Losson,R. (1995) The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. *EMBO J.*, **14**, 2020-2033.
- Lee,J.W., Ryan,F., Swaffield,J.C., Johnston,S.A. and Moore,D.D. (1995) Interaction of thyroid-hormone receptor with a conserved transcriptional mediator. *Nature*, **374**, 91-94.
- Li,Y., Dawson,M.I., Lee,M.-O., Jong,L., Hobbs,P.D. and Zhang,X.-k. (1997) Induction of apoptosis by retinoids in human lung cancer cell lines. *Int. J. Cancer* (in press).
- Liu,Y., Lee,M.-O., Wang,H.-G., Li,Y., Hashimoto,Y., Klaus,M., Reed,J.C. and Zhang,X.-k. (1996) RAR $\beta$  mediates the growth inhibitory effect of retinoic acid by inducing cell apoptosis in human breast cancer cells. *Mol. Cell. Biol.*, **16**, 1138-1149.
- Mangelsdorf,D.J. and Evans,R.M. (1995) The RXR heterodimers and orphan receptors. *Cell*, **83**, 841-850.
- Nagy,L., Thomazy,V.A., Shipley,G.L., Fesus,L., Lamph,W., Heyman,R.A., Chandraratna,R.A. and Davies,P.J. (1995) Activation of retinoid X receptors induces apoptosis in HL-60 cell lines. *Mol. Cell Biol.*, **15**, 3540-3551.
- Onate,S.A., Tsai,S.Y., Tsai,M.-J. and O'Malley,B.W. (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science*, **270**, 1354-1357.
- Pfahl,M. (1993) Nuclear receptor/AP-1 interaction. *Endocr. Rev.*, **14**, 651-658.
- Ponzoni,M., Bocca,P., Chiesa,V., Decensi,A., Pistoia,V., Raffaghello,L., Rozzo,C. and Montaldo,P.G. (1995) Differential effects of N-(4-hydroxyphenyl)retinamide and retinoic acid on neuroblastoma cells: apoptosis versus differentiation. *Cancer Res.*, **55**, 853-861.
- Pratt,W.B. and Toft,D.O. (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr. Rev.*, **18**, 306-360.
- Reed,J.C. (1994) Bcl-2 and the regulation of programmed cell death. *J. Cell. Biol.*, **124**, 1-6.
- Steller,H. (1995) Mechanisms and genes of cellular suicide. *Science*, **267**, 1445-1449.
- Takaoka,A., Adachi,M., Okuda,H., Sato,S., Yawata,A., Hinoda,Y., Takayama,S., Reed,J.C. and Imai,K. (1997) Anti-cell death activity promotes pulmonary metastasis of melanoma cells. *Oncogene*, **14**, 2971-2977.

Takayama,S., Sato,T., Krajewski,S., Kochel,K., Irie,S., Millan,J.A. and Reed,J.C. (1995) Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. *Cell*, **80**, 279-284.

Takayama,S., Bimston,D.N., Matsuzawa,S., Freeman,B.C., Aime-Sempe,C., Xie,Z., Morimoto,R.I. and Reed,J.C. (1997a) BAG-1 modulates the chaperone activity of Hsp70/Hsc70. *EMBO J.*, **16**, 4887-4896.

Takayama,S., Krajewski,S., Krajewska,M., Kitada,S., Zapata,J., Kochel,K., Knee,D., Miller,G., Scudiero,D., Tudor,G., Sausville,E. and Reed,J.C. (1997) Expression and location of Hsp70/Hsc-binding anti-apoptotic protein BAG-1 (HAP-1) and its variants in normal tissues and tumor cell lines. (submitted)

Voegel,J.J., Heine,M.J.S., Zechel,C., Chambon,P. and Gronemeyer,H. (1996) TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO J.*, **15**, 3667-3675.

Wang,H.-G., Takayama,S., Rapp,U.R. and Reed,J.C. (1996) Bcl-2 interacting protein, BAG-1, binds to and activates the kinase Raf-1. *Proc. Natl. Acad. Sci. USA*, **93**, 7063-7068.

White,E. (1996) Life, death, and the pursuit of apoptosis. *Genes Dev.*, **10**, 1-15.

Wu,Q., Dawson,M.I., Zheng,Y., Hobbs,P.D., Agadir,A., Jong,L., Li,Y., Liu,R., Lin,B. and Zhang,X.-k. (1997a) Inhibition of *trans*-retinoic acid-resistant human breast cancer cell growth by retinoid X receptor-selective retinoids. *Mol. Cell. Biol.* (in press).

Wu,Q., Li,Y., Liu,R., Agadir,A., Lee,M.-O., Liu,Y. and Zhang,X.-k. (1997b) Modulation of retinoic acid sensitivity in lung cancer cells by a dynamic balance of nur77 and COUP-TF orphan receptors and their heterodimerization. *EMBO J.*, **16**, 1656-1669.

Yang,Y., Minucci,S., Ozato,K., Heyman,R.A. and Ashwell,J.D. (1995) Efficient inhibition of activation-induced Fas ligand up-regulation and T retinoids requires occupancy of both retinoid X receptors and retinoic ac. *J. Biol. Chem.*, **270**, 18672-18677.

Zeiner,M. and Gehring,U. (1995) A protein that interacts with members of the nuclear hormone receptor family: identification and cDNA cloning. *Proc. Natl. Acad. Sci. USA*, **92**, 11456-11469.

Zhang,X.-k., Hoffmann,B., Tran,P.B., Graupner,G. and Pfahl,M. (1992a) Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. *Nature*, **355**, 441-446.

Zhang,X.-k., Lehmann,J., Hoffmann,B., Dawson,M.I., Cameron,J., Graupner,G., Hermann,T., Tran,P. and Pfahl,M. (1992b) Homodimer formation of retinoid X receptor induced by 9-cis retinoic acid. *Nature*, **358**, 587-591.

Zhang,X.-k. and Pfahl,M. (1993) Regulation of retinoid and thyroid hormone action through homodimeric and heterodimeric receptors. *TEM*, **4**, 156-162.

## Figure Legends

**Figure 1. Inhibition of RAR DNA binding by BAG-1.** (a) Inhibition of RAR/RXR heterodimer binding on the TREP<sub>al</sub> oligonucleotide by BAG-1. In vitro synthesized RAR $\alpha$  and RXR $\alpha$  were preincubated with the indicated molar excess of in vitro synthesized BAG-1 or estrogen receptor (ER). Unprogrammed reticulocyte lysate was used to maintain an equal protein concentration in each reaction. Following this pre-incubation, the reaction mixtures were incubated with <sup>32</sup>P-labeled TREP<sub>al</sub> and analyzed by the gel retardation assay. Open arrow indicates non-specific binding. (b) Inhibition of TR $\alpha$ /RXR $\alpha$  binding by BAG-1. In vitro synthesized TR $\alpha$  and RXR $\alpha$  were preincubated with the indicated molar excess amount of in vitro synthesized BAG-1 and analyzed by the gel retardation assay using the TREP<sub>al</sub> as a probe. (c) Inhibition of RAR $\alpha$ /RXR $\alpha$  binding on the  $\beta$ RARE by BAG-1. In vitro synthesized RAR $\alpha$  and RXR $\alpha$  were preincubated with the indicated molar excess amount of in vitro synthesized BAG-1, and analyzed by the gel retardation assay using the  $\beta$  RARE as a probe. (d) Inhibition of DNA binding of bacterially expressed RAR but not RXR by BAG-1. Bacterially expressed RAR $\gamma$  or RXR $\alpha$  protein was preincubated with 6  $\mu$ l in vitro synthesized BAG-1 and analyzed by the gel retardation assay using the TREP<sub>al</sub> as a probe.

**Figure 2. Analysis of RAR-BAG-1 interaction by the GST pull-down assay.** BAG-1 or a BAG-1 C-terminal deletion mutant (BAG-1/ $\Delta$ c) was expressed in bacteria using the pGex.4T expression vector. The GST-BAG-1 proteins were immobilized on glutathione-Sepharose beads. As a control, the same amount of GST was also immobilized. <sup>35</sup>S-labeled RAR $\alpha$ , RXR $\alpha$  or Bcl-2 was then mixed with the beads. After extensive washing, the bound proteins were analyzed by SDS-PAGE. The input proteins are shown for comparison (left panel). For comparison, binding of GST-BAG-1 and GST-BAG-1/ $\Delta$ c to Hsc70 was shown in right panel.

**Figure 3. RAR and BAG-1 interact in yeast.** The RAR $\alpha$  and BAG-1 cDNAs were cloned into the yeast expression vectors pGAD and pGBT, respectively. The resulting expression vectors were introduced into Y190 yeast cells. The yeast transformants were streaked on a filter and

assayed for  $\beta$ -galactosidase activity (LacZ expression). 1. pGBT11 and pGAD424/BAG-1 (11 + 424/BAG-1); 2. pGBT11/RAR $\gamma$  + pGAD424 (11/A $\gamma$  + 424); 3. pGBT11/RAR $\gamma$  + pGAD424/BAG-1 (11/A $\gamma$  + 424); 4. pGBT9/RXR $\alpha$  + pGAD426 (9/X $\alpha$  + 426); 5. pGBT9/RXR $\alpha$  + pGAD426/RAR $\gamma$  (9/X $\alpha$  + 426/A $\gamma$ ); 6. pGBT9/RXR $\alpha$  + pGAD424/BAG-1 (9/X $\alpha$  + 424/BAG-1); 7. pGBT9 + pGAD426/RAR $\gamma$  (9 + 426/A $\gamma$ ); 8. pGBT9 + pGAD424 (9 + 424).

**Figure 4. Inhibition of trans-activation activities of nuclear receptors by BAG-1.** (a) Inhibition of RAR $\alpha$  activity on the TREpal by BAG-1. The TREpal-tk-CAT reporter plasmid was co-transfected into CV-1 cells with 100 ng RAR $\alpha$  expression vectors together with the indicated amounts of BAG-1 expression vector (pcDNA3/BAG-1) or the empty plasmid (pcDNA3) into CV-1 cells. Transfected cells were treated with or without  $10^{-7}$  M *trans*-RA, and assayed 24 h later for CAT activity. (b) Inhibition of RAR $\alpha$  activity on the  $\beta$ RARE by BAG-1. The  $\beta$ RARE-tk-CAT reporter plasmid was co-transfected with 100 ng RAR $\alpha$  expression vectors together with the indicated amounts of pcDNA3/BAG-1 or pcDNA3 into CV-1 cells. Transfected cells were treated with or without  $10^{-7}$  M *trans*-RA, and assayed 24 h later for CAT activity. (c) Inhibition of RAR $\beta$  activity on the TREpal by BAG-1. The TREpal-tk-CAT reporter was co-transfected by either 100 ng RAR $\beta$  expression vector together with the indicated amounts of pcDNA3/BAG-1 or pcDNA3. Cells were treated with or without  $10^{-7}$  M *trans*-RA. (d) Inhibition of TR activities by BAG-1. The MHC-TRE-tk-CAT reporter plasmid was co-transfected with 100 ng TR $\alpha$  expression vectors together with the indicated amounts of pcDNA3/BAG-1 or pcDNA3 into CV-1 cells. Transfected cells were treated with or without  $10^{-7}$  M T<sub>3</sub>, and assayed 24 h later for CAT activity. (e) Effect of BAG-1 on RXR homodimer activity on the TREpal. The TREpal-tk-CAT was cotransfected with 100 ng RXR $\alpha$  expression vector together with the indicated amount of pcDNA3/BAG-1 or pcDNA3. Cells were treated with or without  $10^{-7}$  M 9-*cis*-RA.

**Figure 5. Over-expression of BAG-1 reduces *trans*-RA-induced growth inhibition of breast cancer cells.** (a) Effect of constitutive BAG-1 expression on *trans*-RA-induced growth inhibition in MCF-7 cells. The growth of BAG-1 stable transfectant, MCF-7/BAG-1(#3), parental MCF-7 cells, and MCF-7 cells transfected with empty vector (MCF-7/neo) in the absence or presence of the indicated concentration of *trans*-RA was determined by the MTT assay. (b) Effect of constitutive BAG-1 expression on *trans*-RA-induced growth inhibition in ZR-75-1 cells. The growth of BAG-1 over-expressing transfectants, 75-1/BAG-1 (#5) and 75-1/BAG-1 (#6), parental ZR-75-1 cells and ZR-75-1 cells transfected with the empty vector (75-1/neo) in the absence or presence of the indicated concentrations of *trans*-RA was determined by the MTT assay.

**Figure 6. Overexpression of BAG-1 inhibits *trans*-RA-induced apoptosis of ZR-75-1 cells.** (a). Inhibition of *trans*-RA-induced apoptosis in ZR-75-1 cells. (b). Inhibition of *trans*-RA-induced apoptosis in MCF-7 cells. Cells were treated with  $10^{-6}$  M *trans*-RA for 48 hours, and DNA fragmentation was determined by the TdT assay. Representative histograms show relative apoptotic cell numbers. FL, fluorescence.

**Figure 7. Over-expression of BAG-1 prevents inhibition of Bcl-2 expression by *trans*-RA in MCF-7 cells.** Cell lysates prepared from MCF-7 and MCF-7/BAG-1 (#3) cells treated with or without  $10^{-6}$  M *trans*-RA for the indicated time were electrophoresed in a SDS-12.5% polyacrylamide gel. After the transfer to nitrocellulose, Bcl-2 was detected with rabbit anti-Bcl-2 serum by Western blotting. For control, expression of  $\alpha$ -tubulin was shown.



— Figure 1a  
Liu et al, 1997

ER:	-	-	-	-	1	2	5
BAG-1:	-	1	2	5	-	-	-
RAR $\alpha$ /RXR $\alpha$ :	+	+	+	+	+	+	+



— Figure 1b  
Liu et al, 1997

BAG-1: - 1 2 5  
TR $\alpha$ /RXR $\alpha$ : + + + +

TR $\alpha$ /RXR $\alpha$  →  
▽



— Figure 1c  
Liu et al, 1997

BAG-1: - 1 2 5  
RAR $\alpha$ /RXR $\alpha$ : + + + +

RAR $\alpha$ /RXR $\alpha$  →



Figure 1d  
Liu et al, 1997

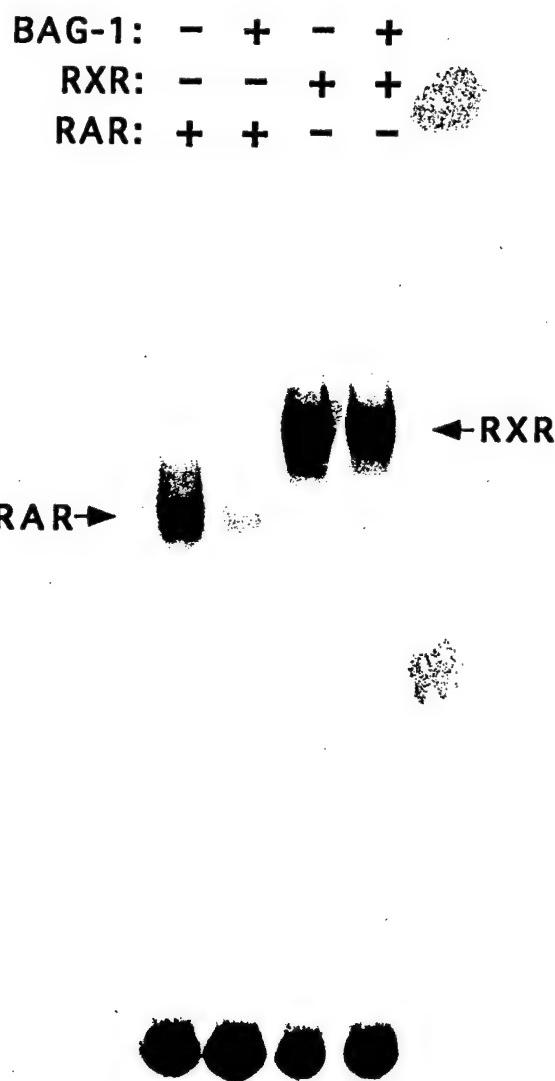


Figure 2  
Liu et al, 1997

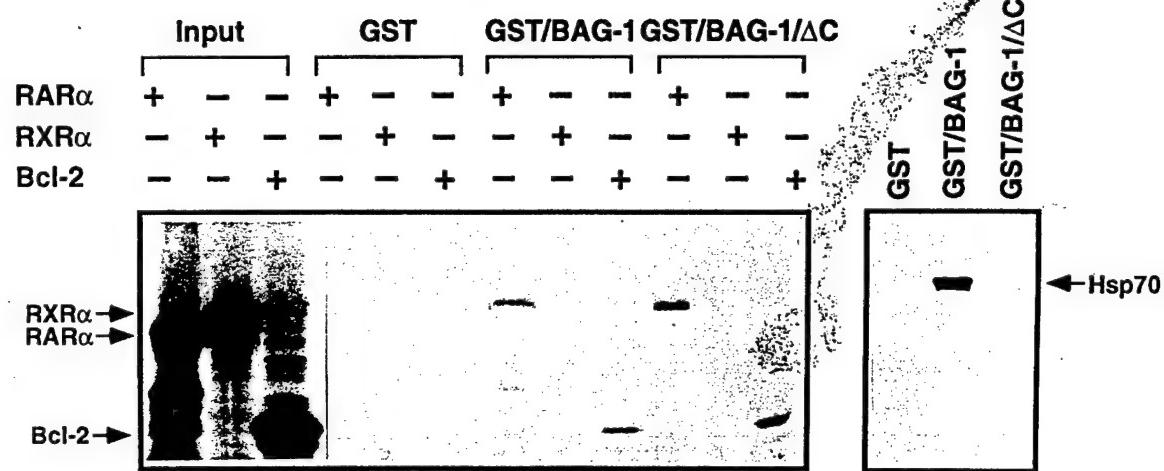


Figure 3  
Liu et al, 1997

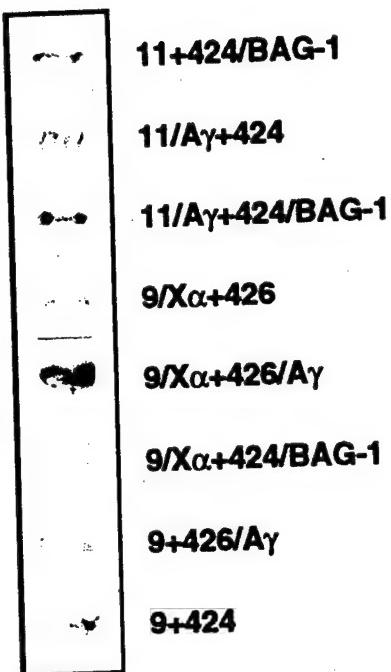
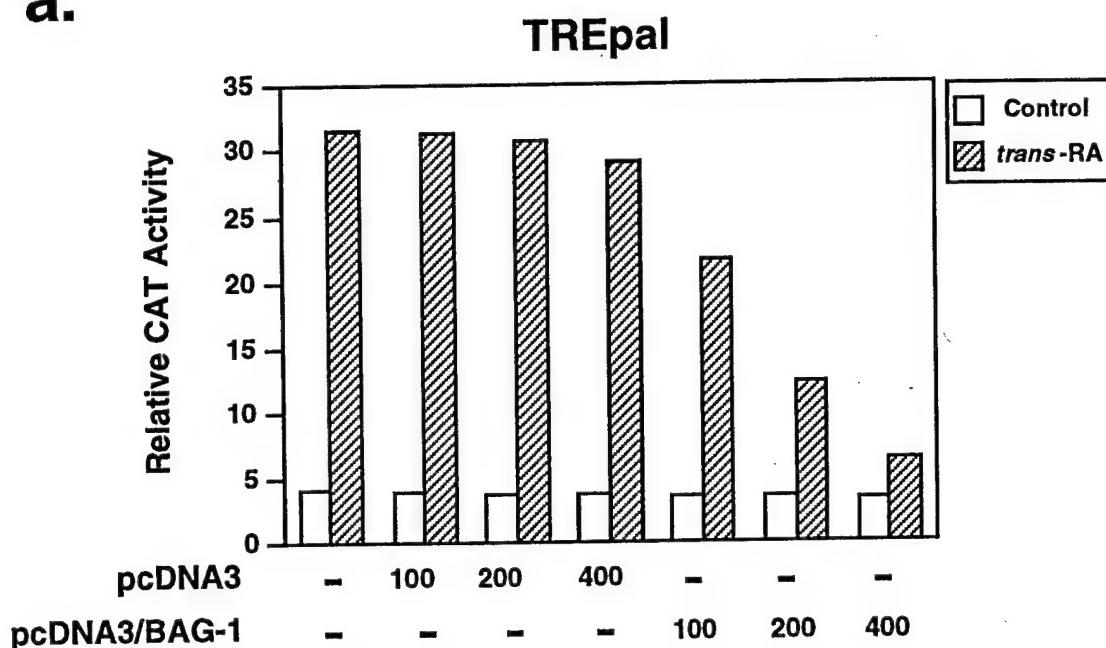
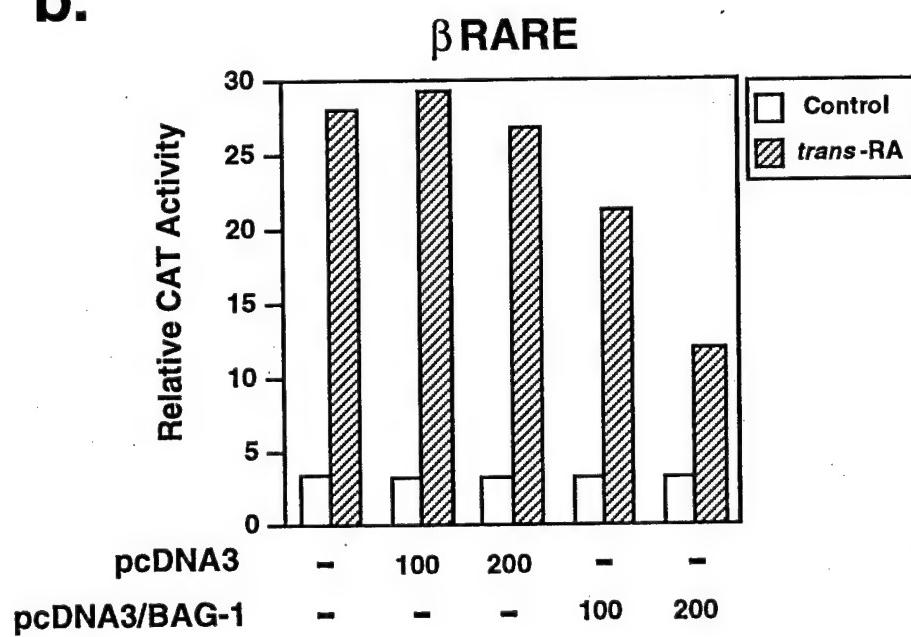


Figure 4a-b  
Liu et al, 1997

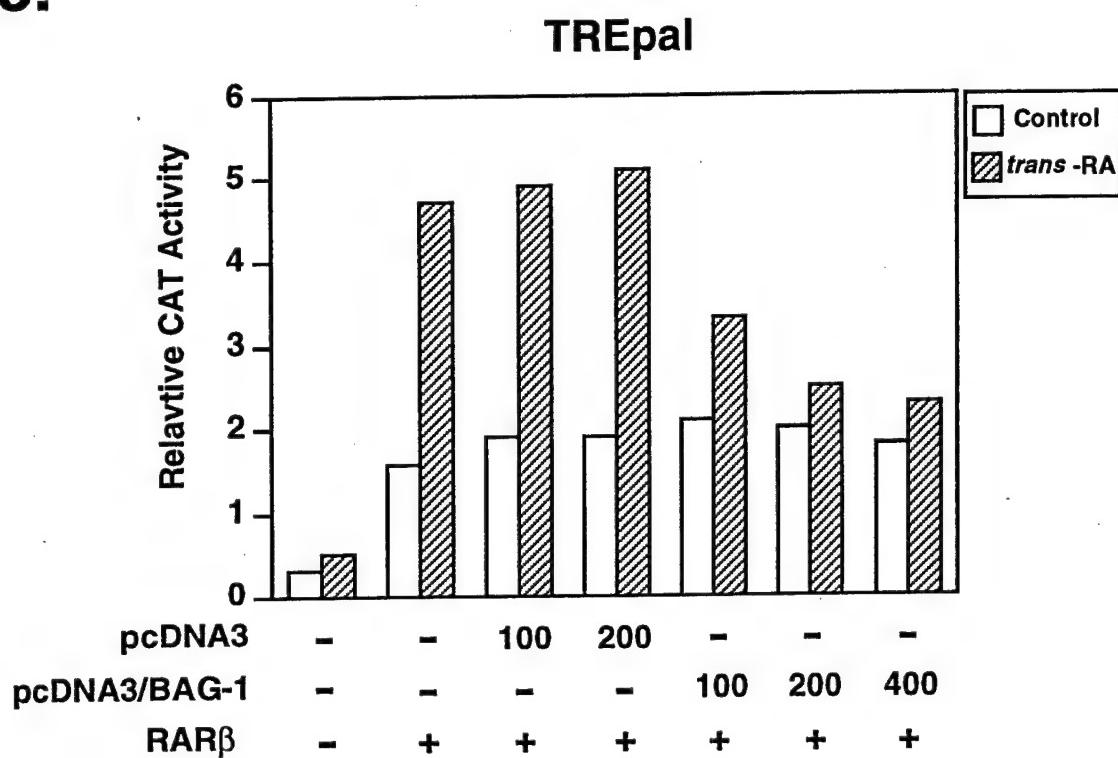
a.



b.



C.



d.

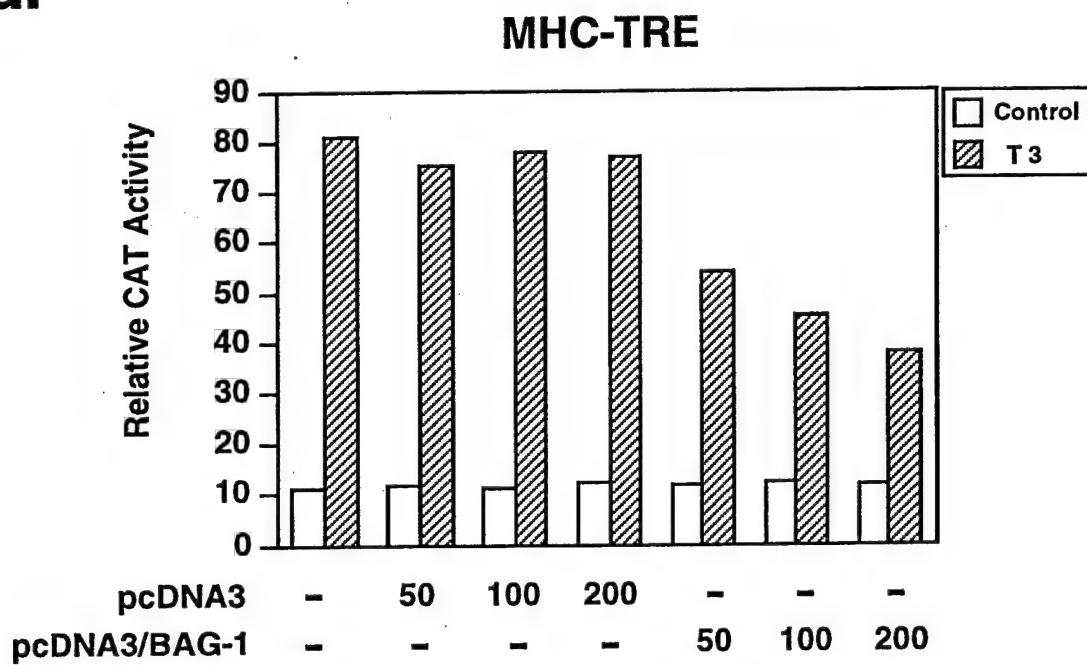


Figure 4e  
Liu et al, 1997

e.

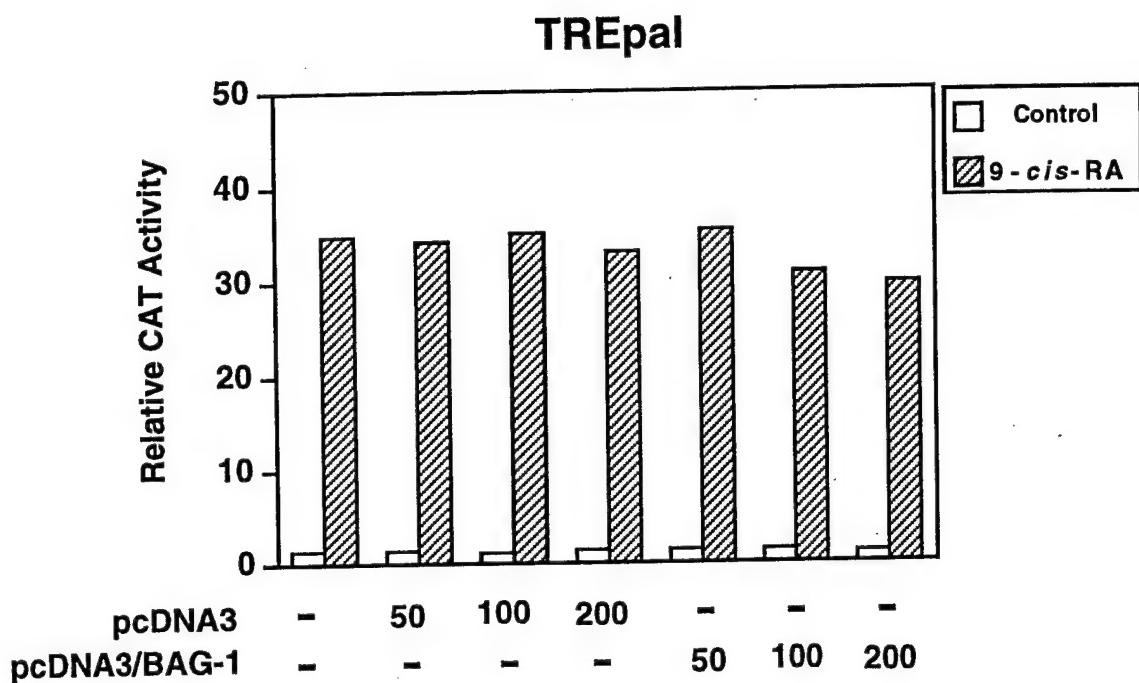
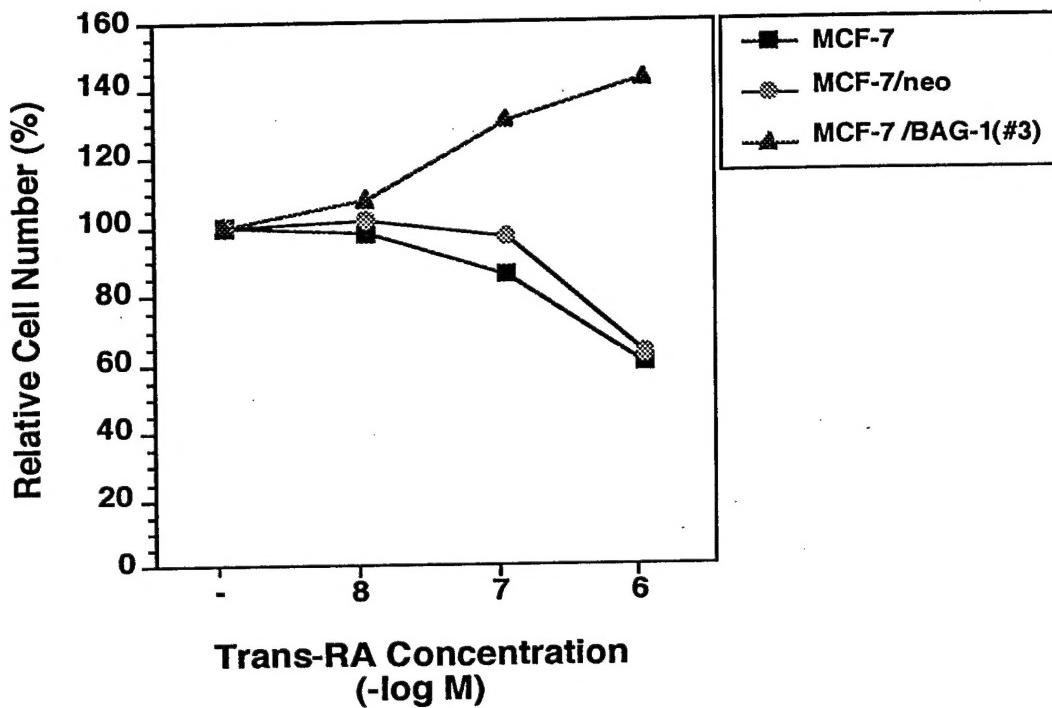


Figure 5  
Liu et al, 1997

a.



b.

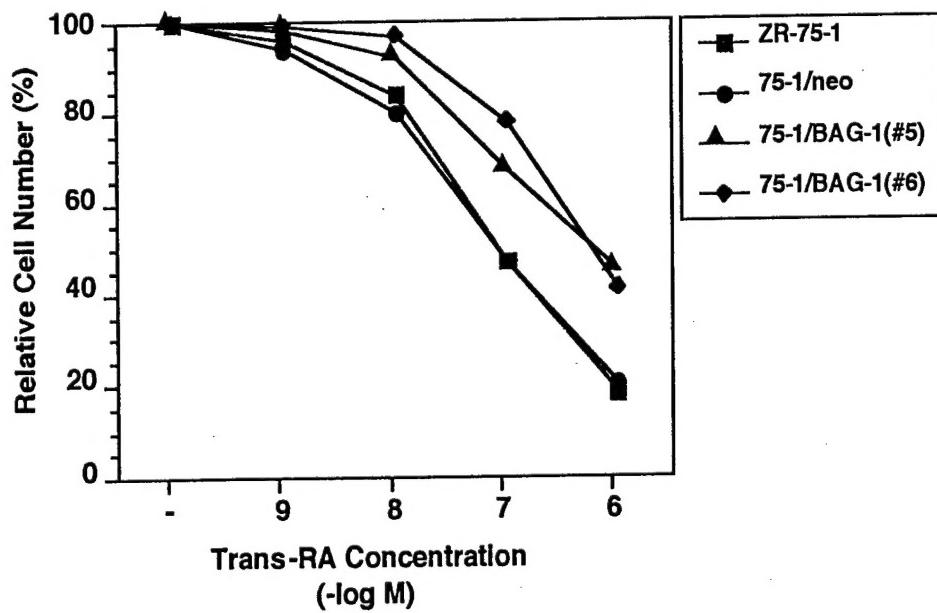
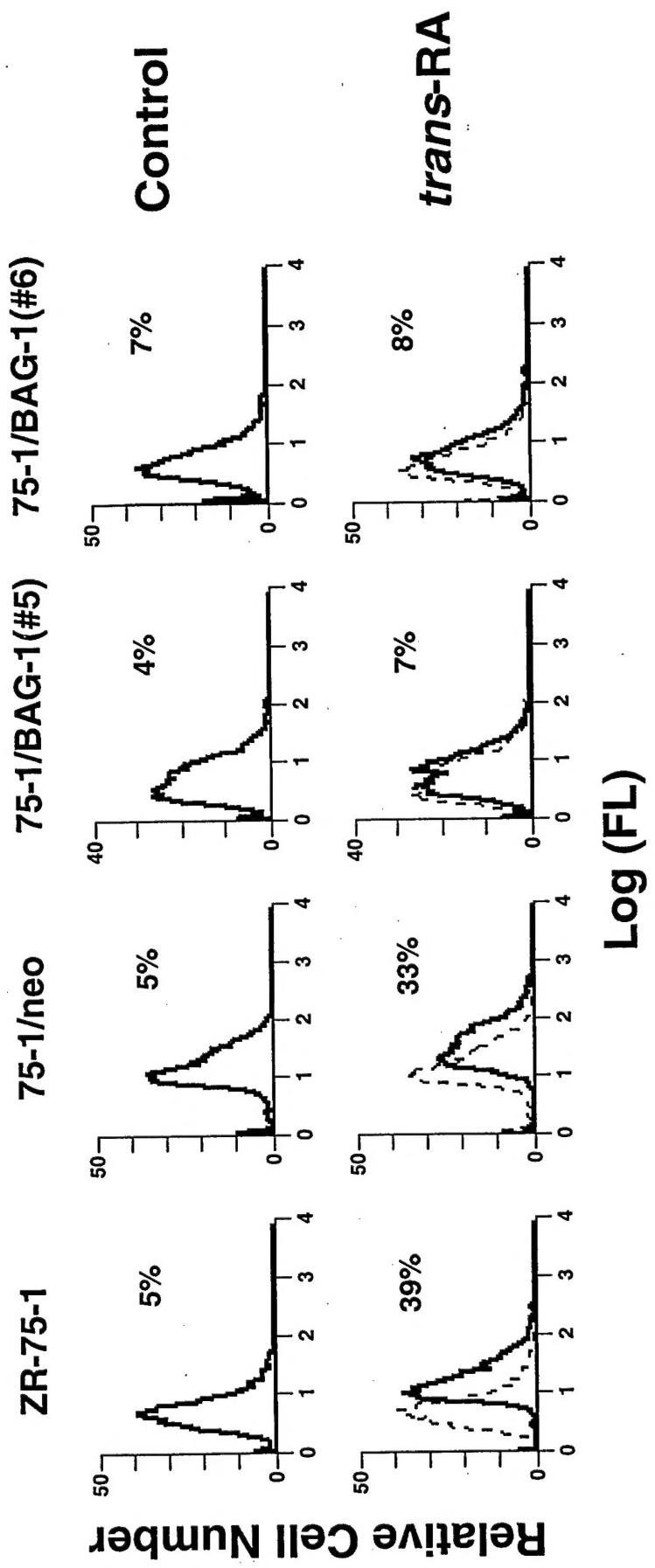


Figure 6a  
Liu et al, 1997



MCF-7      MCF-7/neo

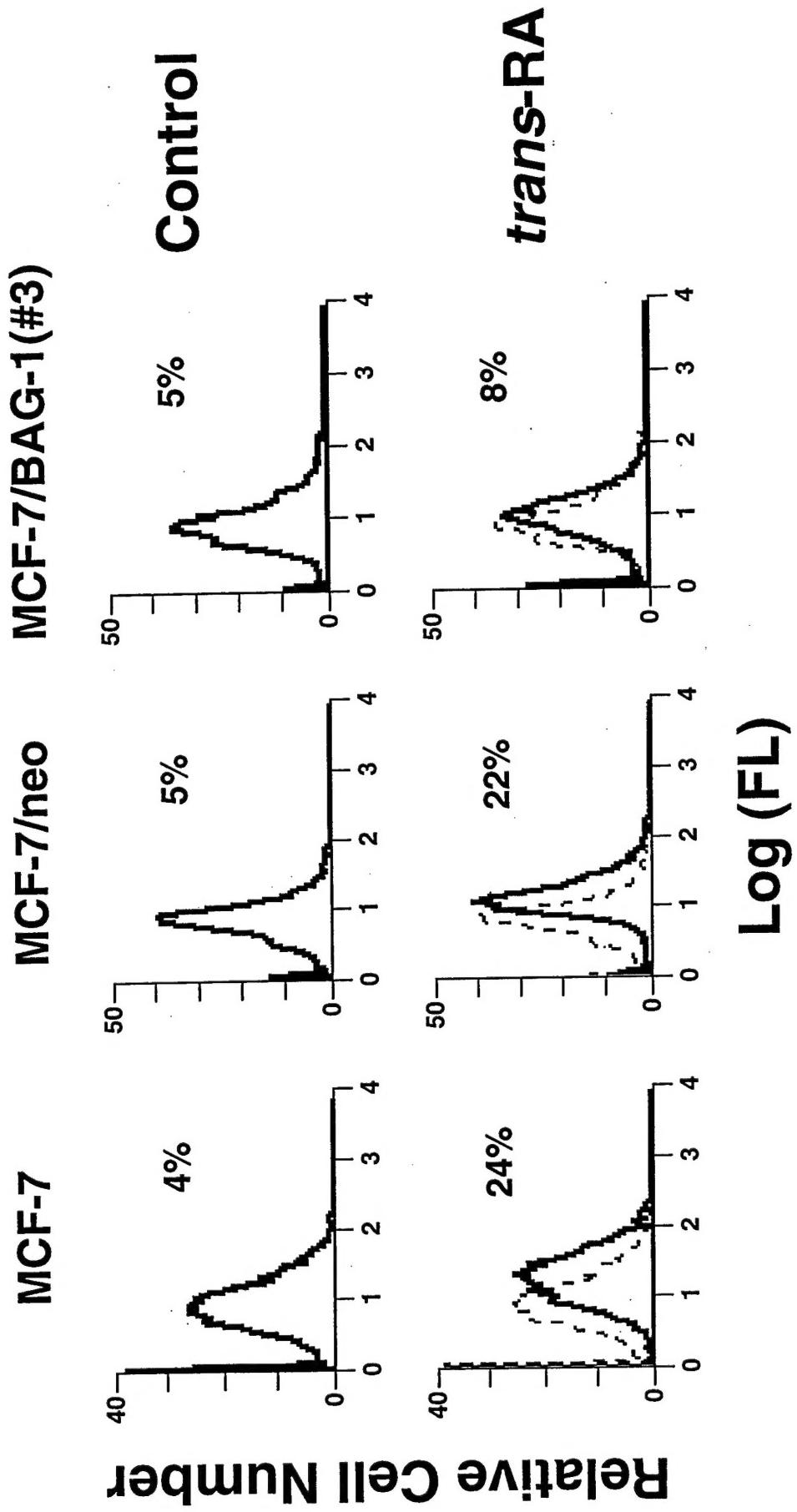


Figure 7  
Liu et al, 1997

